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Duchenne Muscular Dystrophy (DMD) is a lethal X-linked myopathy occurring in 1 in 3000 male births. The gene, which spans over 2.1 kilobases, has been identified and produces a protein designated dystrophin which is an integral component of the cytoskeleton of muscle membrane. In 50 to 60 % of DMD males, the gene defect is a deletion which disrupts the reading frame and results in significantly reduced production or abnormal structure of dystrophin.

The objective of the first phase of this research was to identify deletions in the dystrophin gene in DMD males using a polymerase chain reaction (PCR) procedure. In the PCR, specific oligonucleotides are used to selectively amplify target sites in the gene up to a million fold. In this protocol 9 unique oligonucleotide primers were used in a multiplex PCR to simultaneously amplify sites within the gene known to be deletion prone. Amplification does not occur at the site of a deletion, which results in the absence of a specific band on evaluation by agarose gel electrophoresis.

In this study deletions were identified in 28 of 65 DMD males using the multiplex PCR, which offers a rapid and efficient method of screening for the gene defect. Detection of the site of the deletion in affected males allowed selection of the appropriate cDNA probe for evaluation of female relatives at risk of being DMD carriers. Assessment of gene dosage was made by densitometric quantitation of the restriction fragment band intensities on autoradiograms by comparing females with carrier risk to normal, not deleted controls. While a deletion is easily detected with cDNA probes in males, the presence of a normal X chromosome interferes with visualization of a deletion in a female; therefore the comparison of gene dosage ratios is employed.

37 females representing 21 different kindreds were evaluated with cDNA probes and densitometric analysis of gene dosage. Determination of carrier status was made in 24 females, with 14 females assessed with low and 10 with high carrier probability. Both the PCR and cDNA probe analyses offer greatly improved

reliability in carrier detection by providing direct gene assessment in contrast to earlier indirect methods based on linkage analysis.

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USE OF THE POLYMERASE CHAIN REACTION AND COMPLEMENTARY DNA PROBES IN THE DETECTION OF DUCHENNE MUSCULAR DYSTROPHY CARRIERS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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Dedication

I wish to dedicate this thesis to my mother and father, whose love and encouragement have always provided great inspiration, and to my husband for his love, patience and sense of humor which have sustained me during my graduate program.

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Literature Review

X-Linked Muscular Dystrophy

Clinical Features

Duchenne muscular dystrophy (DMD) is the most common sex linked lethal disease in man and the second most frequent X-linked disorder (1, 2). It is the most frequent and devastating of the common muscular dystrophies with an incidence of 1 in 3500 male births (2, 3).

DMD was originally described by Meryon (4) and Little (5). In 1861 and 1868 Duchenne (6, 7) described the classical infantile form of the disease characterized by pseudohypertrophy of the calves and rapid disease progression. X-linked muscular dystrophy is characterized by progressive muscular weakness and significant degeneration of the skeletal muscle. A typical histological pattern includes widespread degeneration and regeneration of individual muscle fibers in most skeletal muscle groups with subsequent development of extensive connective tissue fibrosis. Usually subclinical symptoms will be seen in the 1 to 3 year age group as delayed motor development (8). Other indicators would eventually include a peculiar

waddling gate, difficulty in climbing stairs and a tendency to frequent falls (9, 10). There is symmetrical weakness and wasting of the muscles first in the proximal muscles of the lower extremities. The calves, and to a lesser extent the deltoids, display pseudohypertrophy as muscle fibers are replaced by connective and adipose tissue. Progressive degeneration continues so that males affected with Duchenne muscular dystrophy are usually confined to a wheelchair by the age of 12 years, with progressive joint contractures leading to spinal deformities (11). Chronic respiratory insufficiency during the second decade of life leads to pneumonia which results in death by the early twenties (1, 2, 3).

Both cardiomyopathy and mental impairment are observed in these patients to varying degrees (1, 12); however, primary cardiac failure is seen as the cause of death in only 10% of patients (13). Mental retardation may be seen even before physical symptoms appear, but the impairment is not progressive. A few patients demonstrate superior intellectual ability, while a small group has an IQ below 50 (1). In a recent study of 80 X-linked muscular dystrophy males investigating the correlation between phenotypic severity with deletion type, no significant correlation was seen between deletion type and IQ (14).

Becker muscular dystrophy (BMD) is a clinically similar but much less severe X-linked myopathy with an incidence of 1 in 30,000 male births (15). BMD is much less progressive

than DMD and some patients remain ambulatory well into their twenties and productive into their forties and fifties. BMD males typically can reproduce, which is especially important since all female offspring will be carriers of the disease. This disease was once believed to map to the long arm of the X chromosome, but is now known to be allelic with the Duchenne muscular dystrophy gene on the short arm of the X chromosome (2). Molecular analysis of deletions has provided proof that the 2 diseases result from mutations in the same gene (1, 2, 16).

Diagnosis

DMD and BMD can usually be distinguished by mode of inheritance and clinical features from rare autosomally inherited dystrophies, such as limb girdle dystrophy, fascioscapulohumeral dystrophy and X-linked Emery-Dreifus syndrome (2). More recent results from molecular investigations indicate that many intermediate forms of the disease exist and strict categorization into DMD or BMD may not always be possible. A more appropriate classification system may be mild, intermediate and severe forms of the disease (17, 18, 19).

High concentrations of muscle enzymes in serum are present in affected males from birth and are observed in a certain percentage of female carriers (20). Among the muscle enzymes and blood proteins which have increased levels are creatine kinase (CK) (21, 22, 23), aldolase (24),

hemopexin (25) and myoglobin (26). These are believed to be secondary manifestations of the disease due to the release from the cytosol of damaged muscle fibers (27). The most dramatic increase in DMD and BMD patients is seen with serum CK, which is also elevated in from 50 to 66% of carriers (28, 29, 30).

Some investigators have focused on the nature of the plasma membrane defect. Verrill et al (31) and others (32, 33) have observed decreased lymphocyte capping in affected males and female carriers with normal values in healthy controls. Erythrocyte membrane studies by Roses et al (34) detected increased phosphorylation of spectrin proteins by endogenous protein kinase in DMD patients.

Female carriers can be classified into 3 groups: definite (also obligate), in which the female has an affected son and a family history of a previously affected male on the maternal side; probable, in which the female has 2 or more affected sons, but no family history; and possible, in which the female has only 1 affected child with no family history (simplex case) or is a sister or other female relative of a muscular dystrophy patient (21, 29).

Due to the random X inactivation, explained by the Lyon hypothesis (35), a wide variation in symptoms is seen in female carriers, which can range from absence of pseudohypertrophy of the calves to the presence of pseudohypertorphy and marked proximal muscle wasting. It is estimated that approximately 8% of carriers exhibit some

clinical manifestation and most show subclinical muscle weakness (36, 37). There are several reports of phenotypically discordant monozygotic twin carriers of DMD which are attributed to uneven X inactivation producing a discrepancy in clinical expression (38, 39, 40). Over 20 cases have been reported in which manifesting females possess an X/autosome translocation, which results in the Duchenne phenotype due to nonrandom X inactivation of the normal X chromosome (41).

Disease Prevalence

There is a high rate of spontaneous mutation, estimated at 4 in 100,000 male births (42). This high rate may be partially explained by the extremely large size of the gene which increases the probability of a mutation and the presence of mutational hot spots (1, 2). DMD males typically do not reproduce and therefore cannot transmit the defective chromosome to the next generation. According to Haldane, if there is an equal mutation rate in both sexes, then 33% of all DMD cases would be expected to arise from spontaneous mutations (43). While this has been supported by some studies (1, 44), a number of investigators have observed a lower proportion of new mutations than predicted by Haldane (0.133, Pickard et al [32], 0.19 to 0.22, Cheeseman et al [45]).

At present there is no cure for the disease. Currently treatment is targeted at alleviation of symptoms, especially

spinal deformities and contractures, and emphasis is on methods of improved diagnosis of affected males and carrier females to prevent disease reoccurrence (1, 2, 3).

Molecular Genetics

The following provides a brief review of the sequence of events which led to the discovery of the dystrophin gene, which when altered results on X-linked muscular dystrophy. As Rowland so elegantly states, "the discovery is the culmination of research that can be called a triumph in reverse genetics" (46, 47). In contrast to traditional genetic approaches in which the gene product is known and the gene may be isolated by applying relatively straightforward protein and recombinant DNA techniques, the gene product in Duchenne muscular dystrophy had not been identified prior to the gene isolation. This increased the difficulty in finding the gene and required implementation of innovative experimental approaches.

Mapping of the DMD Gene

Three lines of evidence contributed to the localization of the DMD gene to the Xp21 locus. Identification and investigation of rare females with DMD or BMD who exhibited a *de novo* X/autosome translocation using high resolution cytogenetic analyses revealed that the Xp21 band was always disrupted (48). These findings made the short arm of Xp21 highly suspect for the DMD gene locus. Additional evidence

placing the DMD gene at Xp21 was provided by linkage analyses in which certain probes mapping to the short arm of the X chromosome (OTC [ornithine transcarbamylase] and L1.28) demonstrated RFLPs (restriction fragment length polymorphisms) which segregated with the DMD and BMD phenotype (49).

Perhaps the most convincing data was the finding of deletions of contiguous genes in the vicinity of the Xp21 locus in several males affected with complex phenotypes which included DMD. Among the additional X-linked disorders associated with DMD were glycerol kinase deficiency, adrenal hypoplasia, retinitis pigmentosa, the McLeod phenotype and chronic granulomatous disease (50). In one of these patients, BB, who had DMD and the last 3 disorders mentioned, cytogenetic analysis revealed a small deletion of portions of bands Xp21.1 and Xp21.2 (51).

Cloning of the DMD Gene

Two different approaches were used to clone the locus. Kunkel et al used DNA from patient BB in competitive hybridization reactions (the phenol enhanced reassociation technique [pERT]) with DNA from an XXXXY male to select for sequences which were enriched for regions deleted in BB. This method was used to isolate 7 clones designated pERT (from the method of isolation) (52). One of these clones, pERT 87 (DXS164) was made available to over 20 research laboratories for use as a molecular probe for RFLP analysis

of males affected with X-linked muscular dystrophy. The results of these studies provided evidence that the pERT locus was tightly linked to the DMD gene and detected deletions in 6.5% of the males evaluated. These data also supported the belief that DMD and BMD were in fact allelic disorders (16).

The second approach utilized one of the female X/autosomal translocations, specifically an X:21 translocation. The exchange point in the X chromosome was in band p21, while in chromosome 21 it was in band p12 which was known to carry tandemly repeated sequences of ribosomal genes. The autosomal exchange point occurred within one of these ribosomal RNA genes and probes derived from this region were used to isolate a larger fragment spanning the X:21 junction. It was predicted that the translocation had disrupted the DMD gene, and that one end of any junction clone should contain the DMD locus. The translocation junction clone, XJ1.1, which resulted from these efforts was used to prepare probes which were linked to DMD detected deletions in about 6% of DMD males as seen for the pERT probes (53, 54).

Using the Xp21 genomic clones van Ommen et al constructed a 4 million base pair map around the DMD gene including a restriction map of the Xp21 region (55). In further mapping studies, the DMD gene was determined to be 2000 kilobases (kb) and a locus was identified and

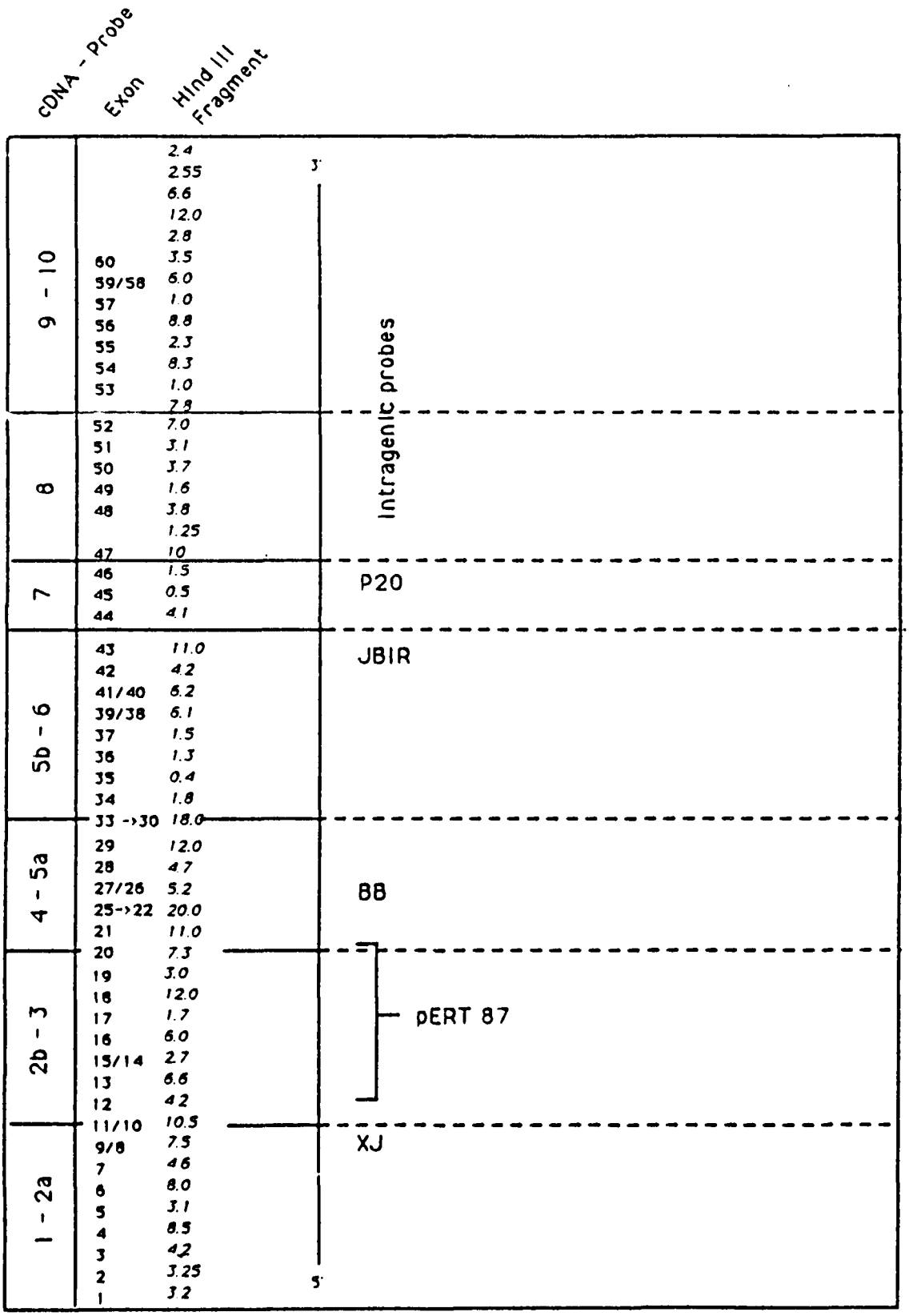
designated J66, which mapped distal within the DMD gene (56).

Monaco et al sequenced portions of the pERT 87 locus and demonstrated interspecies conservation of sequence, including homology with sequences from the mouse X chromosome. They found that one of the human conserved sequences hybridized with a 16 kb transcript isolated from human fetal skeletal muscle and used this sequence to construct cDNA (complementary DNA) clones (57). Subsequently Burghes et al reported the isolation of an adult muscle cDNA clone for the XJ1.1 locus which detected a 16 kb messenger RNA in adult human muscle. They report that subclones from the combined pERT and XJ1.1 clones detected deletions in approximately 11% of DMD patients (58).

Complementary DNA (cDNA) Probes for DMD Gene

In 1987 Koenig et al reported the cloning of the entire dystrophin cDNA corresponding to a fetal skeletal muscle transcript. Figure 1 provides a diagram of the cDNA probes as they relate to other genomic probes and the HindIII restriction fragments identified by these probes. The 14 kb transcript was composed of a minimum of 60 exons which were mapped within Xp21 relative to known loci. The entire gene spanned over 2 million base pairs. This observation indicates a gene nearly 200 times the size of its RNA transcript, with generally small exons averaging 200 base

Figure 1. Location of Intragenic Probes
Relative to Exons



pairs separated by introns averaging 35,000 base pairs. Using the DMD cDNA probes they detected deletions in 50% of 104 affected males with the majority of these concentrated in 2 regions of the gene represented by cDNA probes 8 and 1-2a. While these 2 probes cover less than 2 kilobases of the 14 kilobase cDNA, they detect almost 80% of the deletions observed. These results suggested that certain regions of the gene were more prone to deletion than others (59). More recently den Dunnen et al have determined the size of the gene to be about 2.3 million base pairs containing 75 exons, spaced unevenly throughout the gene, separated by introns ranging from only a few kilobases to 160 to 180 kilobases for the P20 intron (60).

Further evidence for a deletion hot spot in the DMD gene was provided by the isolation of a new intragenic probe, P20, distally located in the gene, which detected deletions in 10% of DMD and BMD patients. This detection rate was higher when compared with other intragenic probes including JBir (7%) and J66 (less than 1%) (61).

Forrest et al screened 66 affected males using a fetal muscle cDNA clone, cf23, which extends from within the pERT region to a region distal to JBir. They observed deletions in 40% of patients using this probe which provided additional evidence for a region of the gene which appears to be preferentially deleted (62). In a subsequent report of 107 DMD patients they detected deletions in 55% using 2

newly constructed probes from a human fetal muscle cDNA library representing only 2.4 kb of the transcript. These were probes cf56a, which corresponds to Koenig probe 8 (59), and cf23a which corresponds to Koenig probe 7. They noted that the majority of DMD and BMD deletions began in different but adjacent regions of the gene which was consistent with Koenig's results. Their data also demonstrated that different exon deletions were associated with either DMD or BMD phenotype, with DMD deletions showing greater heterogeneity in both location and extent (63).

Lindlof et al identified deletions in 50% (45/90) of a DMD patient population in Finland using cDNA probes and found 6 cases with junction fragments. In agreement with previous findings, 84% of the deletions were detected using only probes 8 and 1-2a (64).

A proposal that the pattern of deleted bands is predictive for either the DMD or BMD phenotype was made by Read et al based on deletion analysis of affected males in which a 60% detection rate was observed. They reported greater heterogeneity in both size and location of deletion in DMD than in BMD. Their explanation for these differences centers on the complexity of the dystrophin gene such that there may be a greater number of ways to disrupt the gene and totally abolish function as in DMD, while fewer mechanisms may exist to alter but not severely impair function as in BMD (65).

DMD Gene Product, Dystrophin

In 1987 Hoffman et al identified the product of the DMD gene to be an approximately 400 kilodalton (kD) protein present in relatively low abundance in striated muscle (0.002% of total muscle protein) and in cardiac muscle, smooth muscle and brain. The protein was named dystrophin due to the finding that it was absent or altered in X-linked muscular dystrophy patients. Dystrophin was detected in solubilized tissue by Western blot using polyclonal antibodies directed to an in vitro engineered fusion protein containing 2 portions of the homologous mouse DMD cDNA. Muscle tissue from DMD males demonstrated no detectable dystrophin. These findings were also observed in mdx mice which suggested that these disorders may be homologous (66). The mdx mouse strain is an X-linked murine dystrophy discovered by Bulfield (67). While muscle histology is similar to DMD, the mdx mouse does not develop connective tissue fibrosis and exhibits little if any clinically detectable phenotype (68).

Chelly et al evaluated the 14 kb transcript of the dystrophin gene by a PCR procedure. They identified the transcript in 13 different tissues ranging from 0.02 to 0.12% of total messenger RNA in skeletal muscle to 25,000 times less in lymphoblastoid cells (69). Lev et al reported somewhat different results in studies of the expression of the DMD gene in cultured muscle cells. Using cDNA hybridization assays they detected a 14 kb RNA transcript in

fetal and mature human skeletal muscle but failed to detect this RNA in cultured cells outside the muscle linage (70).

Further investigations of the level of gene expression have been undertaken. Hoffman et al found less than 3% of normal levels or total absence of dystrophin in severe DMD phenotypes, low concentrations in intermediate phenotypes and normal amounts but abnormal molecular weight dystrophin in the mild BMD phenotype. They observed normal levels of dystrophin of normal molecular weight in 20 other neuromuscular disorders evaluated which indicated that dystrophin quantitation may prove helpful in differentiating myopathies which overlap clinically with DMD and BMD (71). In a more recent study Hoffman et al describe the use of dystrophin analysis in evaluation of 97 patients diagnosed as possible BMD. They found 54 to have dystrophin abnormalities while the remaining 43 had normal dystrophin with no X-linked family history (72). Commercial sources for dystrophin testing are available and should soon be offered for carrier testing of females at risk. Unfortunately this test will require muscle biopsies and is predicted to be informative in only 33% of carriers (73).

The primary structure of dystrophin was deduced using the DMD cDNA which has been completely sequenced. The 3685 amino acid sequence appears to form 4 distinct domains which predict a rod shaped molecule. There is homology with both alpha-actinin and spectrin which suggests that dystrophin may be part of a family of cytoskeletal proteins

(74). More recent data obtained in a study of 258 independent deletions at the DMD locus have allowed analysis of the relationship of different dystrophin domains to the pathophysiology of the disease. Interstitial deletion in the N-terminal domain and the first 13 and last 8 repeats resulted in mild disease, while terminal deletions of both the cysteine rich domain and the first half of the C-terminal domain produced more severe disease (75).

Feener et al indicate that the 3′ end of the dystrophin transcript can be alternatively spliced to create a number of isoforms which differ at their carboxyl domain. They propose that these alternative transcripts produce dystrophin molecules which may interact with different proteins in the tissues expressing dystrophin (76).

A number of investigations of X-linked muscular dystrophy have provided evidence that an abnormality exists which impairs function of the cell membrane and that a systemic membrane defect may be responsible for the pathogenesis of the disease (32, 77). Data obtained in studies by Goldsmith and Gruemer, which evaluated drug inhibition of lymphocyte capping, suggested that the differences in capping between affected and normal individuals resided in the membrane itself or in its immediate proximity rather than in the associated cytoskeletal components (78). Recent studies have specifically defined the location of the defect.

The studies by Hoffman et al localized dystrophin to the transverse tubules of triadic junctions in skeletal muscle and reported that it was not associated with the myofibrillar fraction. They postulated a possible role in calcium homeostasis and suggested that it may serve as an anchor protein for binding of actin filaments (79). Watkins et al performed immuno-gold electron microscopic studies which localized dystrophin to the cytoplasmic face of the muscle fiber, possibly on contiguous transverse tubules. They propose a spectrin-like role of dystrophin in membrane stabilization (80). Bonilla et al used anti-dystrophin antibodies in combination with immunofluorescence to demonstrate the presence of the protein at the sarcolemma of normal muscle fibers and the absence in DMD fibers (81). Additional support for sarcolemmal localization is provided by the findings by Zubrzycka-Gaarn et al using antibodies made to synthetic peptides and fusion proteins which demonstrate significant reaction with normal skeletal muscle sarcolemma and reduced or absent immunoreaction with DMD muscle fibers (82).

Phenotypic Variation

There has been keen interest in understanding the reasons for phenotypic differences observed in X-linked muscular dystrophy with respect to the size and location of deletions. Monaco et al published data to support a model based on the effects of deletion breakpoints upon the

translation of triplet codons into amino acids of the protein product. The reading frame theory postulates that in the less severe form of muscular dystrophy the deletion results in maintaining the translational reading frame so that a smaller but partially functional protein is made. In DMD the deletion is proposed to cause a shift in reading frame creating a premature stop codon to produce a truncated, abnormal protein. Evidence for this mechanism was based on determination of sequence for exon-intron borders within the pERT locus where exons had been brought together as the result of a deletion (83). Somewhat different findings were obtained by Malhotra et al who reported that in a number of BMD patients the translational reading frame was not maintained. They suggest that other mechanisms may exist which compensate for the shift in reading frame to produce the milder BMD phenotype. Among those proposed were use of a cryptic promoter, reinitiation of translation from an internal start codon or an altered mode of splicing which might restore the reading frame (84).

Gilgenkrantz et al in a study of 45 deletions found that the reading frame was disrupted in 6 of 7 DMD patients, 1 intermediate and in 3 BMD patients. They note that if compensatory mechanisms exist, they do not appear to work in all cases and may be dependent on the exact location of the intronic breakpoint (19). In a large collaborative study of 258 independent deletions Koenig et al observed that in 92% of cases the correlation between phenotype and type of

deletion is in agreement with the reading frame theory. They propose that this information may be useful in disease diagnosis and prognosis. These authors suggest that there may be many "in frame" deletions in the DMD gene which are not detected because the individual is asymptomatic or has atypical clinical features (75). Kunkel et al speculate that given the cardiac complications seen in X-linked muscular dystrophy some of these silent deletions may account for a proportion of patients being treated for cardiomyopathies (84).

Liechti-Gallati et al promulgate the concept that within the hot spot region a phenotype prediction can be made from knowledge of the breakpoint locus of an observed deletion and the number of exons involved. Their results agreed with the reading frame theory (85). Further support for the reading frame theory came from Gillard et al who found only 2 exceptions to the theory in a study of 38 patients based on sequence analysis of exon-intron borders (15).

In addition to deletions other types of gene alterations have been identified. Bettecken and Muller used field inversion gel electrophoresis of SfiI restriction fragments hybridized to the intronic probe JBir to identify a 220 kb insertion present in a family with an atypical course of muscular dystrophy. The insertion was seen in 2 of 3 affected males (one deceased) and 2 sisters which were determined to be carriers based on these data (86). There

have been a few reports of other mutations such as gene duplications (87, 88, 89). In a recent study den Dunnen et al identified duplications in 13 of 115 deletions which represents 10% of the total mutations seen. In this study the evidence for duplication appears to be based on visual assessment of increased hybridization intensity of restriction fragments identified by cDNA probes rather than using analytical techniques (60).

Since only 50 to 60% of mutations in the gene are deletions, a large percentage of nondeletion mutations in the DMD gene may be mutations in the splice site. These may be point mutations or small deletions which would potentially alter or prevent splicing of introns to yield the mature messenger RNA. This might produce a deletion of a single exon or possibly inclusion of an intron (91).

Carrier Detection

Linkage Analysis. The availability of an abundance of both flanking and genomic probes for the DMD gene offered the opportunity to make carrier determinations in female relatives of affected males. Earlier methods of carrier detection used linkage studies based on the segregation of genetic markers, particularly RFLP's, with the disease gene.

In 1983 Harper et al used probes RC8 and L1.28 which flank the DMD locus to make genotype predictions in DMD families. Because these probes were located at a moderate distance from the DMD gene (RC8, 15 centiMorgans and

L1.28,13 centiMorgans) and were subject to a greater chance of crossover, they were not appropriate for prenatal diagnosis (90).

With the isolation of intragenic probes more accurate carrier diagnosis was possible. Darras et al used 12 different probes, both intragenic and flanking, to evaluate carrier status by RFLP analysis. While results demonstrated successful predictions in the majority of cases, at least 1 affected male fetus was misdiagnosed due to crossover (91).

Bartlett et al reported the use of 13 Xp21 RFLP probes which covered the entire length of the DMD gene to screen affected males for deletions. The combination of probes detected deletions in 39% of the patients screened. In one family study where all affected males were deceased, a deletion was demonstrated in 3 generations of females by virtue of the segregation of the grandfathers' RFLPs in contrast to the null alleles inherited from the hemizygous carrier females (92).

In a more recent study Prior et al presented a series of family studies using RFLP probes for DMD carrier detection which included a discussion of the advantages and limitations of this method. In addition to requiring an affected male in order to identify which restriction fragment segregates with the normal and mutant gene, the study usually requires the analysis of many family members. This is complicated in cases of adoption or questionable paternity. As for other X-linked RFLPs, the mother must be

informative (heterozygous) for a marker to be useful, and there is the possibility of phase reversal by crossover between the marker and the mutation in 2 to 4% of meioses. Furthermore the accuracy of carrier status in isolated cases is limited by the probability that the affected male may be the result of a new mutation (93).

cDNA Probe Analysis. With the development of cDNA probes for the dystrophin gene diagnosis and carrier detection were greatly facilitated. Since intragenic deletions account for 50 to 60% of mutations (59) and these can be detected by cDNA probes in both affected males and carrier females, the reliability of carrier diagnosis should be improved. Additionally cDNA analysis should be more efficient since fewer probes are needed as compared to RFLP's and testing of extensive pedigrees is avoided.

One of the first applications of cDNA probes in prenatal diagnosis in families affected with DMD was reported by Darras et al. In one case the family was uninformative by the RFLP approach and a male fetus appeared to have a high risk of being affected based on RFLP linkage data. Direct assessment of the gene by cDNA probe analysis demonstrated a deletion in the living affected male which was not found in the male fetus or the mother. Based on these results the pregnancy was continued since the affected male appeared to be a spontaneous mutation and the mother was determined to have low probability of being a carrier.

While the interpretation was based on comparison of restriction fragment intensities, there was no indication as to what methods were used for quantitation (94).

In 1988 Darras and Franke published a standard pattern of restriction fragments which may be detected with HindIII or BglII restriction digests of normal human DNA using 7 cDNA subclones representing the entire 14 kb DMD cDNA. They also reported 12 restriction site polymorphisms (95). In a companion paper they report detection of 21 deletions in 32 X-linked muscular dystrophy patients in hybridization studies with DMD cDNA probes. In 4 of these deletions they identified novel restriction fragments (junction fragments) which may be very useful in diagnosis of carrier females. They present results of carrier detection using RFLP probes based on relative fragment intensities by densitometric measurement of bands on autoradiograms; however, no comparable data was provided for carrier assessment using cDNA probes. They were able to determine the meiotic or mitotic origin in 13 of the 21 families and observed germline mosaics in 3 cases (96).

Germline mosaicism is believed to account for the recurrence of several human disorders that are produced by autosomal dominant or X-linked mutations, for example DMD and lethal osteogenesis imperfecta (1, 97). This phenomenon is the result of a dual population of gametes, some with the mutation and some without it. Evidence that a germline mosaic exists may be seen in cases of multiple offspring

affected with the disorder while the parents appear to be normal. Bakker et al described two DMD families in which the mothers showed no deletion with pERT probes in their somatic cells but transmitted the disease to two children. The deletions in both offspring were identical indicating that this was probably an early mitotic event in germline proliferation (98). Darras and Franke reported a family with a history of DMD in which 2 sisters received a deletion from their father who was phenotypically normal. The deletion was not present in the mother who would have been considered an obligate carrier according to conventional pedigree analysis. The origin of the mutation was made using haplotype analysis with nine informative RFLP markers (99). Additional reports of germline mosaics in up to 10% of families (60, 96) emphasize that they may make a substantial contribution in the induction of DMD mutations. It is recommended that carrier determinations should be performed on the sisters of affected males who present as isolated cases even though there is no deletion found in the mother. It is suggested that prenatal analysis should be offered to all potential carriers of this type (99).

Speer et al describe use of cDNA probes cf23a and cf56a to make a prenatal diagnosis of an unaffected fetus in a female DMD carrier, since the deletion observed in the affected male was not seen in the fetus. In another family they were able to trace a deletion through 3 generations in a BMD pedigree which demonstrated conserved inheritance of

identical deletions over many generations. They mention that there is risk of error in prenatal diagnosis using cDNA probes to detect deletion due to the high frequency of new mutations in this gene. They suggest that identification of deletion breakpoints, indicated by junction fragments, is optimum since it circumvents the problems of performing dosage estimates on restriction fragments (100). Unfortunately the majority of deletion breakpoints within the DMD gene are intronic and so will not be detected by the cDNA probes which are derived from exonic portions of the gene (86).

Mao and Cremer investigated the carrier status of 18 females in 6 unrelated DMD kindreds by gene dosage analysis. The restriction fragments which are deleted in the affected male are predicted to show a reduced intensity in female carriers who would be hemizygous for these fragments, while noncarriers would exhibit normal band intensity. The analysis requires that at least one band within a cDNA probe is present to provide an internal control for comparison to the putative deleted band. Using dosage analysis they found 33% of females tested to be carriers (101). It is assumed that dosage analysis was based on visual assessment of band intensities, since no data are presented to suggest quantitative measurements.

Using the concept of dosage analysis Prior et al have described a refinement of the basic method by quantitating autoradiographic bands using densitometry. Carrier

determinations were made by comparison of band ratios of potentially deleted bands to nondeleted bands between females at risk and normal controls. The advantages of such analyses would be to decrease subjectivity inherent in visual assessment of band intensities and so increase the accuracy of gene dosage analysis (102).

While cDNA analysis offers greatly improved accuracy in diagnosis, the Southern protocol is extremely labor intensive requiring a large investment of time and use of radioisotope. This method is not efficient for evaluation of a large population, since affected males may need to be tested with up to 7 cDNA subclones to locate a deletion. There was a need for a more practical approach to screening affected males for deletions prior to carrier analysis.

Polymerase Chain Reaction (PCR)

Methodology. The development of PCR (polymerase chain reaction) methodology has revolutionized molecular biology. Because PCR is a relatively rapid and straightforward method, it offers significant technical advantages over the Southern protocol; however, the real power of the technique is its versatility with potential application in virtually all scientific disciplines. The PCR, which was developed by Mullis et al at Cetus corporation, is used to amplify specific segments of DNA that lie between two regions of known sequence. The amplification is accomplished using oligonucleotide primers which are complementary to sequences

flanking the area of interest and a thermostable DNA polymerase (103).

The synthetic cycle consists of 3 basic steps: denaturation of template DNA by high temperature incubation, annealing at a lower temperature to allow primer binding to complementary target sequences of template DNA and extension of primer: DNA template in a 5′ to 3′ direction using a thermostable DNA polymerase. The cycle is repeated 25 to 40 times, with each successive cycle doubling the amount of desired DNA product, since products of one amplification cycle serve as templates for the next round. Using the PCR selective amplification of a discrete region of a gene can be amplified up to a million fold from nanogram quantities of genomic DNA. The degree of sensitivity is such that a single copy of a genomic sequence is sufficiently amplified to allow detection by Southern hybridization (104, 105, 106, 107).

Mutation Analysis by PCR. Once genomic sequences are amplified, they may be analyzed for the presence of mutations by a variety of methods. Saiki et al described one of the first applications of the PCR as it was used in the diagnosis of sickle cell anemia to amplify beta globin genomic sequences. The amplified target DNA was hybridized to end-labeled probes and evaluated by the oligomer restriction method to distinguish the normal beta A allele from the beta S (sickle) allele (108).

phenylketonuria (114) and beta thallasemia (115). Since the genetic defect in these conditions results from point mutations, the techniques applied to them have not been useful in detecting DMD mutations which are primarily deletions observed in 50 to 60% of cases. The enormous size of the gene and the heterogeneity of deletions offered a unique challenge to devise a rapid and comprehensive method of screening males with X-linked muscular dystrophy.

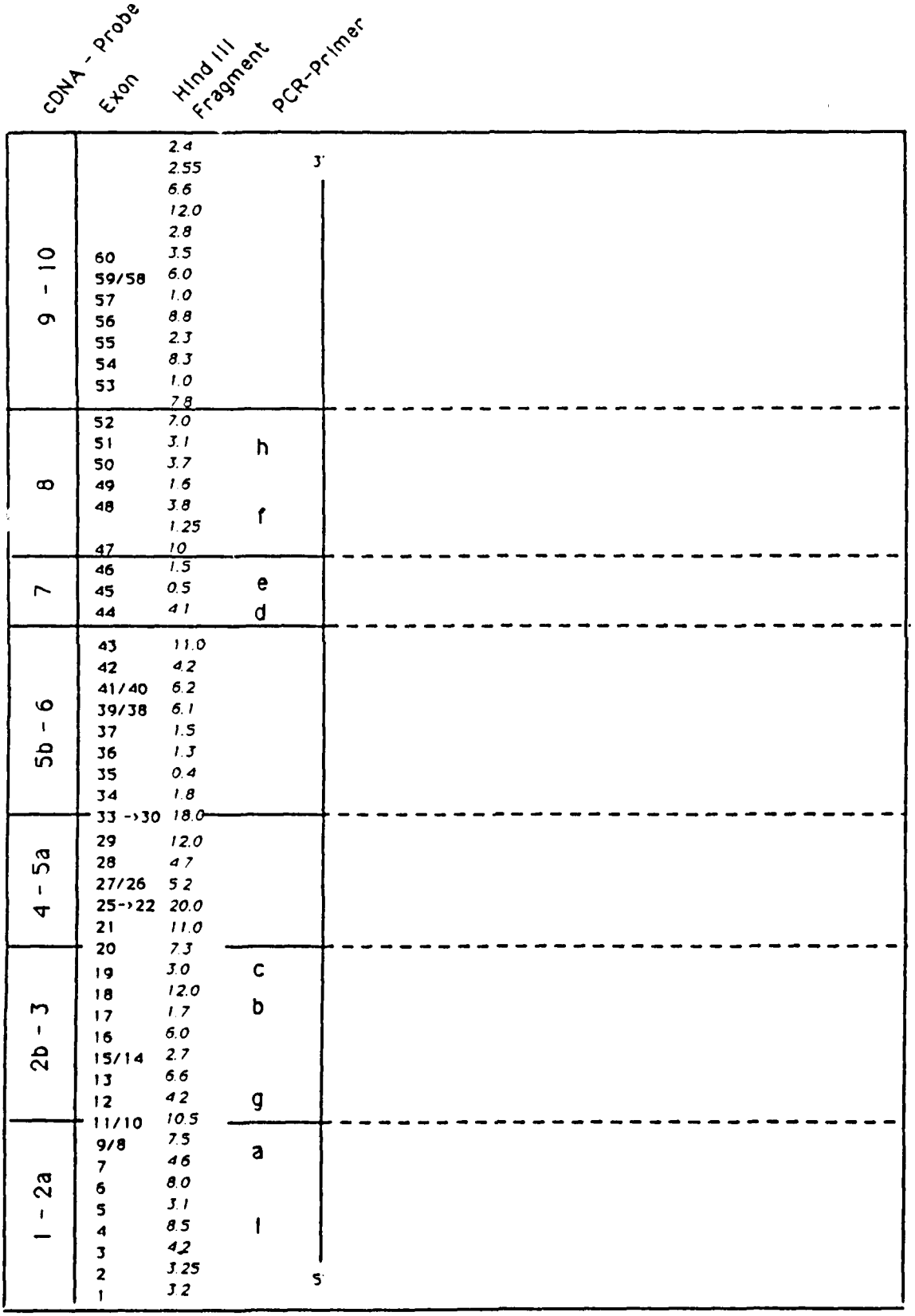
Deletion Analysis in DMD by PCR. While these deletions are heterogeneous in both extent and location, they seem to be concentrated in two regions of the gene, in cDNA probe 7 and 8 and cDNA probe 1-2 (58-64). Chamberlain et al took advantage of these deletion prone regions in the development of a multiplex screening protocol to amplify multiple sites within the gene simultaneously. In contrast to cases where the mutation site is known or in small genes, analysis of a large gene where the mutation site has not been identified is more complicated. The exons which were most often deleted were sequenced to provide the information needed to design oligonucleotide primers to target the exon-intron borders of interest (116). Using a modification of reaction conditions by Kogan et al in the detection of hemophilia mutations (113) Chamberlain developed a system for coamplification of 6 widely separated regions of the dystrophin gene in a single PCR procedure. The multiplex method was reported to detect deletions in 37% of patients

or 70% of the deletions found with DMD cDNA probes. Additional primer sequences were published by Speer et al (117) in the region of cDNA probe 8 and Chamberlain et al (104) in the region of cDNA probes 1-2. At this time the multiplex has been extended to 9 separate sites within the DMD gene as shown in Figure 2 and is predicted to increase detection to 90% of the deletions seen with cDNA probes (104, 116).

In a more recent report, Hentemann et al describe the use of an abbreviated PCR screen using 2 primers in the regions of cDNA probe 1b and probe 7 (118). They observed deletions in only 16% of patients which indicates the limited utility of such a method as compared to a 9 primer multiplex which detects over 50% more deletions.

Future applications of the PCR for the DMD gene may include multiplex sequencing or heteroduplex mapping to identify point mutations which has been used for the HPRT (hypoxanthine-guanine phosphoribosyl transferase) locus (104, 107). PCR may also be useful in the investigation of splice site mutations. If the exons are missing from the mature messenger RNA transcript even though they are present in the genomic DNA, their absence would be predicted to result in abnormal sized PCR products for a specific region of the transcript (84). Protocols are being developed which apply the multiplex PCR method using limited amplification cycles for haplotyping potential DMD carrier females. Since there is quantitative representation of

Figure 2. Location of PCR Primers in the DMD Gene



starting material in the PCR throughout the reaction cycle, females heterozygous for a deletion should demonstrate a two fold reduction in DNA template and therefore only half the yield of a specific amplified fragment. Implementation of such a method to quantitate gene dosage could be employed in routine screening of females for heterozygous gene rearrangements to determine carrier status (107).

Materials and Methods

Specimen Collection

Blood specimens were collected from patients and their families at the following Muscular Dystrophy Association clinics: Children's Hospital, Richmond, Virginia; Kluge Rehabilitation Center, University of Virginia, Charlottesville, Virginia; Children's Hospital, Norfolk, Virginia.

For DNA analysis 30 to 40 ml of blood was collected by venipuncture into acetate-citrate-dextrose (ACD) tubes and for serum creatine kinase (CK) 5 to 10 ml of blood was collected into a red vacutainer tube. The blood was maintained at room temperature until processed, usually within 5 hours of collection. Samples not collected in MDA clinics were shipped by overnight express mail and were processed immediately upon receipt. A complete family pedigree was obtained for use in data interpretation.

DNA Isolation

Contents of each ACD tube were treated individually to lyse cells and recover the nuclear pellet. Blood was transferred to a 50 ml polypropylene tube and to each tube

an equal volume of 2X sucrose triton (0.64 M sucrose, 0.02 M Tris base, 0.01 M $MgCl_2$, 2% Triton X-100, pH 7.6) was added and the tube shaken. Twenty-five ml of 1X sucrose triton was added to the initial mixture to complete lysis. Reaction mixtures were then placed on ice for 10 minutes with mixing by inversion several times during the incubation. Lysates were centrifuged at 12,000 g at 4°C for 15 minutes. The supernatant was decanted and the tube drained briefly before transferring the nuclear pellet to a 1.5 ml sterile microfuge tube. The pellet was then frozen at -70°C or transferred to a clean 50 ml polypropylene tube for digestion with proteinase K.

Nuclear pellets were resuspended in a lysing solution containing 200 microliters 10% sodium dodecyl sulfate (SDS), 20 microliters 20 mg/ml proteinase K (Boehringer Mannheim Biochemicals) and 1.8 ml STE (0.1 M NaCl, 0.01 M Tris, 0.01 M disodium EDTA [ethylenediamine tetracetic acid], pH 8.0). The mixture was incubated in a 37°C water bath overnight (16-24 hours). After incubation the tubes were centrifuged for 10 minutes at 9,000 g to recover condensate on the tube wall.

The proteinase K digest was extracted with 2 ml PCI-9 (100 g phenol, 100 ml chloroform, 1 ml isoamyl alcohol, 10 ml 0.05 M Tris [pH 9.0], 10 ml deionized water] by inversion 10 to 12 times followed by centrifugation at 15,000 g for 10 minutes at 4°C. The aqueous phase was carefully removed to a clean tube using a wide bore disposable pasteur pipette

without disturbing the protein debris at the interface with the lower organic phase. The aqueous phase was then reextracted with 2 ml SEVAG (24 parts chloroform: 1 part isoamyl alcohol) and centrifuged at 15,000 g for 10 minutes at 4°C. The aqueous phase was transferred to a clean polypropylene tube containing 10 ml cold ethanol (stored -20°C until use) and swirled gently to precipitate the DNA. The DNA precipitate appeared as a white, gelatinous mass and was removed from the ethanol by capture on the tip of a Pasteur pipette which had a closed and slightly hooked tip (prepared by flaming). The DNA was allowed to air-dry briefly (3 to 5 minutes) and then transferred to a 1.5 ml sterile microfuge tube containing 200 to 400 ml TE buffer (0.01 M Tris HCl, 0.001 M disodium EDTA, pH 8.0) depending on the size of the precipitate. The DNA samples were stored at 4°C.

Measurement of DNA Concentration

Quantitation of DNA concentration was performed on samples which had been allowed to dissolve at least overnight in TE buffer and were warmed at 37°C for 30 minutes prior to making the dilution for measurement. A 100-fold dilution was prepared using 10 microliters DNA in 990 microliters of TE buffer. The solution was mixed gently with a Pasteur pipette and transferred to a quartz cuvette. Absorbance readings at 260 and 280 nm were made on a Cary 118 or Perkin Elmer lambda 3 spectrophotometer. A TE buffer

blank was used to zero the instrument. The DNA concentration was calculated by the following formula:

A_{260} X dilution X 50 micrograms/ml X volume of sample = micrograms DNA

A 260/280 ratio between 1.8 and 2.0 is indicative of DNA of high purity and minimal protein contamination. If the ratio is not within this range, the sample may be reextracted.

Multiplex Polymerase Chain Reaction (PCR)

Deletion screening was accomplished using a modification of a multiplex PCR protocol originally described by Chamberlain et al (116). This procedure employs multiple, unique sets of oligonucleotide primers directed to specific regions of the gene and a thermostable DNA polymerase to selectively amplify areas of interest. The presence of a deletion is indicated by the failure of the primer to anneal and therefore amplify at specific sites. This results in the absence of bands from PCR reaction products corresponding to specific primers.

The basic components of the multiplex PCR include template DNA, reaction buffer, deoxynucleotide triphosphates (dNTPs), primer sets (forward and reverse) and a thermostable DNA polymerase.

In order to avoid DNA contamination all glassware was acid-washed and sterilized by autoclaving. All reagents

were prepared with double deionized water and sterilized by autoclaving. Pipettors used for PCR determinations were maintained exclusively for this purpose. These pipettors were never allowed to contact amplified reaction mixtures. The laboratory bench was maintained as clean as possible with frequent changes of laboratory mats and gloves.

Primer Synthesis and Preparation

The oligonucleotide primers were synthesized by the Center for Innovative Technology, Medical College of Virginia, Richmond, Virginia, using the phosphoramidite method on an Applied Biosystems Model 380 DNA synthesizer. The nucleotide chain is attached to a solid support during synthesis which allows easy removal of excess reagents by filtration.

There are 5 basic steps involved in the addition of each new nucleotide. DNA is synthesized in a 3′ to 5′ direction with 1 of the 4 nucleotides (dATP, dCTP, dGTP or dTTP) attached to the support. Step 1 is the removal of the dimethoxytrityl group from the terminal nucleoside to free the 5′ hydroxyl group. The second step is an activation reaction in the presence of tetrazole which creates a highly reactive phosphoramidite derivative of the next nucleoside which, in turn, reacts with the 5′ hydroxyl. The coupling reaction which follows is extremely rapid with the displacement of the diisopropylamine group from the phosphoramidite derivative and formation of a 5′ to 3′ internucleotide

linkage through the trivalent phosphorus of the derivative. The product of the coupling reaction is an unstable phosphite triester which is oxidized to a stable pentavalent phosphate triester in the presence of an iodine-water-lutidine-tetrahydrofuran solution. Following oxidation one cycle of the nucleotide addition is completed and the newly created 5′ terminus of the oligomer is protected by a dimethoxytrityl group which will be removed during the next cycle.

The primers are prepared as either forward or reverse members of a set depending on which direction they will anneal to the template strand. Each primer is received in concentrated ammonium hydroxide and is initially heated at 55°C overnight to remove the trityl group. The primer is then concentrated to dryness for 4 to 6 hours in a Savant Speedvac with a dry ice trap. The dried residue is resuspended in sterile double deionized water and diluted to make absorbance readings at 260 nm. The DNA concentration was calculated using the following formula for oligonucleotides under 50 base pairs (119):

$$A_{260} \text{ X } 20 \text{ micrograms/ml X dilution X volume} = \text{micrograms DNA of sample}$$

The primer concentration was adjusted to achieve a final stock concentration of either forward or reverse primer of 50 micromolar. When used in the final reaction

volume of 100 microliters, 1 microliter of each primer will result in 0.5 micromolar final reaction concentration or 1 micromolar for each primer set. Reconstituted primer solutions are stored at 4°C while dried primer stocks are maintained at -20°C. Table 1 provides a list of primer sequences, gene location, and size of amplification product.

Multiplex PCR

The 10X reaction buffer contains Tris HCl (670 millimolar), ammonium sulfate (166 millimolar), disodium EDTA (67 micromolar), $MgCl_2$ (67 millimolar), and 2-mercaptoethanol (100 millimolar). Reaction mixes are prepared in a large volume master mix and then aliquoted to reaction tubes. These may be prepared the day of the run or prepared in advance and frozen at -20°C. Preparing large volumes of master mix at one time and freezing aliquots increases efficiency and uniformity from run to run. Each 100 microliter multiplex reaction mix contains 10 microliters 10X reaction buffer, 15 micrograms bovine serum albumin (3 microliters of 5mg/ml stock, Sigma, fraction V), 10% (w/v) dimethoxy-sulfoxide (DMSO) using 9 microliters stock DMSO/100 microliters reaction volume, double deionized water to achieve a final reaction volume of 100 microliters, 10 microliters each of dATP, dCTP, dGTP and dTTP (10 millimolar stocks, United States Biochemical Corporation), 2 microliters of each primer set premixed as described below, 5 units of Taq polymerase (Amplitaq recombinant Taq

Table 1

Sequences of Oligonucleotide Primers for PCR Multiplex Screen

Primer	Primer Sequence[1]	Gene Location[2] (Probe /Exon)	Amplified Product (bp)[3]
a	F-GTCCTTTACACACTTTACCTGTTGAG R-GGCCTCATTCTCATGTTCTAATTAG	16/8	360
b	F-GACTTTCGATGTTGAGATTACTTTCCC R-AAGCTTGAGATGCTCTCACCTTTTCC	3/17	416
c	F-TTCTACCACATCCCATTTTCTTCCA R-GATGGCAAAGTGTTGAGAAAAAGTC	3/19	459
d	F-CTTGATCCATATGCTTTTACCTGCA R-TCCATCACCCTTCAGAACCTGATCT	7/44	268
e	F-AAACATGGAACATCCTTGTGGGGAC R-CATTCCTATTAGATCTGTCGCCCTAC	7/45	547
f	F-TTGAATACATTGGTTAAATCCCAACATG R-CCTGAATAAAGTCTTCCTTACCACAC	8/48	506
g	F-GATAGTGGGCTTTACTTACATCCTTC R-GAAAGCACGCAACATAAGATACACCT	2/12	331
h	F-GAAATTGGCTCTTTAGCTTGTGTTTC R-GGAGAGTAAAGTGATTGGTGGAAAATC	8/51	409
i	F-TTGTCGGTCTCCTGCTGGTCAGTG R-CAAAGCCCTCACTCAAACATGAAGC	1a/4	196

[1]Primer sequences are shown in 5′ to 3′ orientation. F: Forward primer which hybridizes 5′ of the exon; R: Reverse primer which hybridizes 3′ of the exon.

[2]Indicates the gene location amplified by specific primers in reference to cDNA probes which hybridize to the exons shown.

[3]Indicates the size of the amplification product obtained with each set of primers.

polymerase, Perkin Elmer Cetus, Norwalk, CT, 5U/microliter) and 1 to 2 microliters of template DNA to be amplified.

In contrast to the original method (116) which included all primer sets in one reaction, the protocol was modified to use 2 separate PCR reactions for each DNA sample. For example, with the 6-plex screening procedure, 2 reactions each containing 3 primer sets were used. One 3-plex contained the primer sets e, b, and a while the other 3-plex contained primer sets f, c and d. For the 9-plex screen, 2 primer sets were used as a 4-plex and a 5-plex. The 5-plex contained primer sets e, b, a, g and i while the 4-plex contained f, c, h and d. While this arrangement resulted in setting up an additional reaction mix for each sample, it provided superior resolution of bands and facilitated interpretation. Primers were premixed before addition to the reaction mixture using equal amounts of forward and reverse primer from each set. These primer sets were mixed to create multiplexes which might contain 3, 4 or 5 primer sets each.

The master mix is prepared and includes all reaction components except the Taq polymerase and template DNA. On the day of a run the master mix was either prepared fresh or aliquots thawed. The amplification enzyme, Taq polymerase, was stored at -20°C until use and was maintained on ice during use to prevent loss of activity. One microliter containing 5 units enzyme was used in each multiplex reaction mixture (5 units for each 3-, 4- or 5-plex).

The DNA template was added last, with caution so as to prevent contamination between tubes. Usually 1 microliter of sample was added to provide approximately 1 microgram of DNA for amplification. The reaction will proceed with less DNA (down to 200 nanograms); however, there are occasional samples where it is necessary to increase the amount since they may have lower concentrations than were originally determined. To minimize contamination at this point gloves were changed frequently. The reaction mix was mixed gently by thumping the tube and overlaid with 3 drops of sterile light mineral oil.

Each run includes a reagent control containing all reactants except template DNA, a normal nondeleted control and a deleted control (in whom the deletion was confirmed by complementary DNA [cDNA] studies). These controls were incubated under the same conditions as test samples. The amplification reaction was performed in an Ericomp thermocycler. This instrument contains blocks which are alternately heated or water-cooled to achieve the programmed temperature conditions. There are 2 blocks, block 2 which has 29 positions (for 0.6 ml tubes) and block 1 with 19 positions (for 1.5 ml tubes), available. One position in each block contains a tube with a thermistor to monitor the temperature of reactants. Each well within the block was filled with 3 drops of mineral oil and the reaction tube positioned carefully to ensure good contact with the well. The following reaction conditions represent 1 reaction

cycle: denaturation of template DNA at 94°C for 2 minutes, annealing of primers at 55°C for 2 minutes and extension at 70°C for 3 minutes. The final cycle differs in that it increases the 70°C extension to 10 minutes as the final step. The amplification procedure requires about 5 hours in block 2 and 7.5 hours in block 1. Separate controls were included in each block. After the last cycle reaction products were removed immediately and stored at 4°C until evaluated by electrophoresis.

Electrophoresis of PCR products

PCR products were separated using high voltage electrophoresis on 2.5 to 3% agarose gels. Within the 9-plex, amplification products range in size from 196 to 547 base pairs. Preliminary experiments using lower concentrations of agarose did not provide sufficient band resolution.

Gels were prepared by dissolving agarose (NuSieve GTG, FMC Products) in 1X TBE buffer (90 millimolar Tris, 90 millimolar boric acid and 2 millimolar disodium EDTA) by boiling. For each gel 80 ml of agarose was poured (after cooling to 55°C) into a 9 x 13 cm gel-casting tray. Two 10-tooth, 1 mm gel combs were inserted into the gel, one at the top and one midway in the gel to create two wells in each vertical lane. This provided a well for each of the 2 PCR reactions set up on each DNA sample within one vertical

lane, which facilitates interpretation of bands. Gels were cooled at room temperature for at least 1 hour and then stored in 1X TBE buffer at 4°C until use.

Each gel was placed in a horizontal slab gel electrophoresis chamber (IBI Apparatus) with an EC452 power supply in approximately 1000 ml of 1X TBE with the origin placed at the cathode to allow migration of the negatively charged DNA toward the anode. Twenty microliters of each PCR reaction mixture were transferred to a clean microfuge tube. Air bubbles were expelled to remove mineral oil from the pipette tip before aspirating the sample. The tip was cleaned with a Kimwipe to remove any remaining mineral oil before expelling contents. Two microliters of tracking dye (33% glycerol, 7% SDS and 0.07% bromphenol blue in water) were added and contents mixed. Twenty microliters of each reaction mix were loaded into either the upper or lower well for a given patient. A 123 base pair DNA ladder (BRL, Bethesda Research Laboratories Life Technologies, Gaithers- burg, MD) was used on each gel as a molecular size marker. The gel was electrophoresed at 100 volts for 2.5 to 3 hours until the dye band had migrated approximately 4 cm from the origin.

After electrophoresis the gels were stained for about 30 minutes in 1X TBE with ethidium bromide (0.5 micrograms/ml). Caution was taken to avoid skin contact, since ethidium bromide is a known mutagen. This dye intercalates between the DNA strands and fluoresces upon ultraviolet exposure which allows visualization of the DNA present.

Preliminary experiments with ethidium bromide incorporated in the gel resulted in retarded mobility and band distortion. It was determined that addition of ethidium bromide with continued electrophoresis at low voltage also produced band distortion, possibly due to cathodic migration of the dye molecules.

Gels were destained in deionized water for 30 minutes to 1 hour. Staining and destaining times will vary from gel to gel. After sufficient destaining to clear the background, gels were placed on a Spectroline TS-302 ultraviolet transilluminator for visualization of the bands. The gels were photographed with a polaroid MP-4 land camera using Polaroid type 667 film to record the band patterns and to obtain a permanent record for the presence or absence of deletions.

cDNA Analyses

Restriction Endonuclease Digestion of DNA

DNA was digested with HindIII restriction endonuclease (BRL). For each sample 10 micrograms of DNA was digested using 30 Units of HindIII, 5 microliters 10X BRL React 2 buffer and double deionized water to achieve a final volume of 50 microliters. The digestion mix was incubated in a 37°C water bath for 4 hours and 5 microliters of bromphenol blue tracking dye were added. If the digests were not evaluated immediately, they were stored at -20°C. The restriction digests were previewed for completion of

digestion by electrophoresing 5 microliters of the digest on a 25 ml minigel (0.8% agarose in 1x TBE) for 1 hour at 100 volts. In samples where digestion was not complete, the sample may be further incubated at 37°C.

In preparation for hybridization experiments the digested samples were separated by low voltage electrophoresis on 0.8% agarose gels (9 X 13 cm) overnight (14 to 16 hours). Gels were prepared by melting agarose in 1X TBE buffer by boiling. Wells were formed by using a 10-tooth, 2 mm gel comb. For each gel 65 ml of agarose was poured (after cooling to 55°C) in a 9 X 13 cm gel-casting tray and allowed to cool for at least 1 hour. At this time a small amount of 1X TBE was placed around the comb to facilitate removal. The gel was stored in 1X TBE at 4°C until use.

Forty-five microliters of digested sample including tracking dye was loaded in each well. A 1 kilobase (kb) DNA ladder (BRL) was included on each gel as a size marker in order to monitor relative band migration and to ensure retention of the 5 kb fragment which was needed in dosage analysis with certain probes. Each gel included at least 1 lane for a HindIII digested normal control (nondeleted). Gels were electrophoresed in a horizontal slab gel chamber at a constant voltage of 20 to 25 volts for 14 to 16 hours at room temperature. Gels were stained with ethidium bromide (0.5 micrograms/ml) for 15 minutes and photographed to document fragment migration. A fluorescent ruler was used to label the distance migrated for subsequent

interpretation. The top left edge of the gel was notched to provide orientation during Southern transfer.

Southern Transfer

An upward Southern blot procedure was used to transfer DNA restriction fragments separated by gel electrophoresis to a nylon filter. By denaturing the DNA under alkaline conditions, it is rendered single stranded with subsequent transfer to the nylon filter (119, 120).

Some gels were depurinated prior to alkaline denaturation. Depurination of DNA fragments 15 kb or larger should facilitate transfer to the nylon filter. As judged from the photographs, the top portion of the gel from the origin to about the 10 to 15 kb band location was immersed in 0.25 M HCl. This step was accomplished by placing the gel, still resting on the casting tray, at about a 30-degree angle into the depurination solution for 15 minutes. In this way exposure of smaller fragments to the depurination solution was avoided, which minimized loss during transfer. The gel was rinsed in deionized water for 2 minutes before transferring to the denaturation conditions.

The gel was placed in approximately 250 ml 1X denaturing solution (0.5 N NaOH, 0.6 M NaCl) for 1 hour with slow rotation. An upward Southern transfer system was prepared. A clean sponge (11 X 13 cm) was saturated with denaturing solution and placed in a large dish filled with 1X denaturing solution. A wick of blotting paper cut the

same size as the gel was prewet with denaturing solution and placed across the sponge to extend into the denaturing solution on either end of the sponge. A piece of filter paper prewet with denaturing solution was placed on top of the wick. Bubbles were removed at each step with a clean glass rod or test tube. The gel was then placed underside oriented up on top of the filter paper. The nylon filter (Oncor) was prepared by prewetting with deionized water for 2 minutes and soaking in denaturing solution for 15 minutes. The nylon filter was then placed with the positively charged side down on top of the gel and a prewet filter paper and 2 pieces of blotting paper were placed on top of the nylon filter. A 6-inch stack of dry paper towels, cut the size of the filter, were placed on top with a glass plate with a 500 gram weight applied to distribute pressure evenly. The transfer assembly was covered with Saran Wrap to prevent evaporation while transfer proceeded overnight (18 to 24 hours) at room temperature.

After the transfer was completed, the system was dismantled with the dehydrated gel and nylon filter removed together in order to mark the positions of the origin on the filter. A date and unique label was used on each filter for identification purposes. The filter was washed in neutralizing solution (0.5 M Tris, 1.0 M NaCl) for 15 minutes on slow rotation to remove gel fragments. The filter was removed and blotted between filter paper and allowed to air dry for at least 30 minutes. It was then

exposed to ultraviolet light for 15 seconds on the Spectroline transilluminator to enhance fixing of DNA.

The filter was stored in protective plastic document protectors until used for hybridization. The collapsed gel was stained with ethidium bromide (0.5 micrograms/ml) in 1X TBE for 30 minutes and then destained with deionized water. The gel was viewed on the UV transilluminator to check for remaining DNA which may not have transferred and also indicated other problems in transfer such as the presence of bubbles.

Labeling of cDNA Probes

DNA probes for the dystrophin gene were obtained as freeze dried plasmids from the Repository of Human DNA Probes and Libraries American Type Culture Collection, Rockville, MD. The following probes were used in hybridization studies: cDMD 8 (ATCC # 57674) and cDMD 5b-7 (ATCC # 57672). These plasmids were amplified in alac Iq host, Escherichia coli XL1 Blue (Stratagene) in Dr. Eric Westin's laboratory at the Medical College of Virginia, Richmond, Virginia. After amplification, the plasmid DNA was recovered and probe inserts were excised with EcoR1.

Probe 7 was excised from probe 5b-7 by restriction digest with HincII (BRL). The resulting fragments, 5b-6 and 7 were separated on a preparative gel (1.5% low melting agarose in 1X TBE). The band containing the probe was cut out of the gel and divided into small sections for

resuspension in water in a ratio of 1 part gel to 3 parts water. The probe in agarose was stored at -20°C and used in random primer labeling reactions.

The random primer method originally developed by Feinberg and Vogelstein (121) was employed to radio label the cDNA probes. A mixture of synthetic hexanucleotide primers is hybridized to the DNA to be labeled and the complementary strand is synthesized from the 3′ hydroxyl termini of the primer, using the Klenow enzyme. The reaction mix contains 3 unlabeled deoxynucleotides (dATP, dGTP, dTTP) while the dCTP is labeled with ^{32}P. One of the advantages of this method over the nick translation method is increased efficiency of labeling since priming of DNA synthesis occurs at numerous sites along the denatured DNA.

The cDNA probes were labeled using the Amersham Multiprime DNA Labeling System (RPN.16012). For cDNA probe 8, 120 nanograms of probe was transferred to a 1.5 ml microfuge tube and heat denatured by boiling for 2 minutes. The reaction components were added in the following order: ten microliters of buffer solution 1 (dATP, dTTP and dGTP in Tris HCl [pH 7.8], MgCl, and 2-mercaptoethanol), 5 microliters primer solution 2 (random hexanucleotides in aqueous solution with BSA), a volume of double deionized water to achieve a final reaction volume of 50 microliters, 5 microliters ^{32}P dCTP (DuPont NEN) at a specific activity of 3000 Ci/mmole, and 2 microliters enzyme solution

(1 Unit/microliter DNA polymerase, Klenow fragment in 50 millimolar potassium phosphate [pH 6.5], 10 millimolar 2-mercaptoethanol and 50% glycerol). The reaction mix was gently mixed and centrifuged for 15 seconds to ensure all reagents were in the bottom of the tube. The reaction mixture was incubated for 2.5 to 3 hours at room temperature. Because of the high level of incorporation, probes labeled using this method were used directly in blot hybridizations without separation from unlabeled nucleotides.

If the probe was to be used immediately, it was denatured by boiling for 2 minutes and briefly cooled on ice before addition to the prehybridization solution. Otherwise the probe was stored at -20°C until use. ^{32}P labeled probes were used within 2 weeks from labeling due to the relatively short half-life of ^{32}P (13.7 days).

cDNA probe 7, which was recovered in agarose, was labeled in a similar procedure as probe 8 with the exception that 50 nanograms of probe was used in a reaction volume of 100 microliters. After denaturation, the probe was cooled at room temperature rather than on ice to avoid congealing of the agarose prior to the labeling reaction and addition to the prehybridization mixture.

Hybridization Protocol

The nylon filter bearing the DNA fragments was prepared prior to hybridization with the labeled probe. Prehybridization blocks the nonspecific attachment of the probe to the surface of the filter which can create high background. The prehybridization solution was converted to the hybridization solution by addition of the labeled probe. The conditions for prehybridization and hybridization as described below were low stringency (high ionic strength, low temperature) to maximize annealing between the probe and its target sequences bound to the nylon filter.

Prehybridization buffer of 2X strength (300 millimolar NaCl, 30 millimolar Na citrate) was prepared by mixing the following components in the order shown: prewarm 500 ml 20X SSC (3 M NaCl, 0.3 M Na citrate) and add 13.8 gm $NaH_2PO_4 \cdot H_2O$); add 20 ml prewarmed 10% SDS; add approximately 200 ml deionized water; add 100 ml 50X Denhardt's solution (1% w/v ficoll, 1% w/v polyvinylpyrrolidine, 1% w/v BSA) over low heat with stirring; continue heating to dissolve the precipitate which forms by adjusting the pH to 6.5 with 10 N NaOH. The solution should clear as pH nears 6.5. Adjust the final volume to 1 liter and place the solution in a plastic container before adding 250 mg yeast RNA, dissolving over low heat. Storage in plastic is necessary, since yeast RNA adheres to glass surfaces. The 2X prehybridization buffer was stored at room temperature until use. To prepare 1X prehybridization buffer (150 millimolar

NaCl, 15 millimolar Na citrate), warm the 2X solution for 30 minutes by mixing over low heat to dissolve the precipitate which forms at room temperature. Remove 20 ml of 2X prehybridization buffer and add 20 ml formamide (IBI) which has been prewarmed to 42°C.

The filter to be hybridized was wet with 6X SSC (900 millimolar NaCl, 90 millimolar Na citrate) and placed in the 1X prehybridization buffer in a plastic dish so that the entire surface of the filter was covered. The filter was prehybridized overnight (16-24 hours) at 42°C in a shaking water bath set at low speed. Shorter prehybridizations usually resulted in high background which makes quantitation of band intensities difficult.

The previously labeled probe, which had been denatured by boiling 2 minutes, was cooled briefly and then added to the prehybridization buffer by adding approximately 500 microliters of 1X prehybridization buffer to the probe before pipetting into the hybridization chamber. Following addition of the labeled probe the dish was carefully rocked back and forth to ensure adequate mixing. The hybridization reaction was allowed to proceed overnight (16 to 24 hours) to encourage maximum annealing between the probe and its target sequences.

After hybridization the filters were washed to remove excess probe using conditions of increasing stringency (lower ionic strength and higher temperatures) as described below. These conditions should remove all but the most

complementary probe: DNA hybrids. The washing steps were critical in this protocol since quantitative assessment of band intensities requires very clean autoradiograms free of background signal.

The filter was removed from the hybridization buffer and briefly rinsed in a small volume of wash solution 1 (2X SSC [300 millimolar NaCl, 30 millimolar Na citrate], 0.5% SDS] to remove the majority of unbound label. The filter was placed in 250 ml of fresh wash solution 1 for 10 minutes at room temperature and then transferred to 250 ml of wash solution 2 (2X SSC, 0.1% SDS) for 15 minutes at room temperature. Wash 3 (0.1X SSC [15 millimolar NaCl, 1.5 millimolar Na citrate], 0.5% SDS) was performed at 60°C for 1 hour in a shaking water bath followed by a fresh solution of wash 3 for 30 minutes to 1 hour at 60°C. The filter was rinsed briefly in 0.1X SSC before briefly blotting between filter paper to remove the shiny surface but not to complete dryness. If the filter was completely dried, it was very difficult or impossible to remove the probe to perform rehybridization with another probe. The filter was placed in a plastic document protector and sealed to prevent drying during autoradiography.

Autoradiography

In a darkroom, the filter was placed between 2 sheets of Kodak XAR-5 film in a DuPont Cronex X-ray cassette with QuantIII intensifying screens. The filter was taped in

place on the bottom film to avoid shifting when the top film was removed. The exposure times varied from 3 to 10 days. The double film method allowed viewing the film at 2 to 3 days and continuing exposure if additional time was needed. The exposed film was developed using a Kodak RP X-Omat processor.

Densitometric Analysis of Restriction Fragment Intensities

Female relatives of MD patients known to have a deletion were evaluated for carrier status by determining the relative band intensities of restriction fragments detected by the cDNA probe from the area of the deletion. To establish dosage ratios there had to be at least 1 not deleted restriction fragment in the affected male which would be equivalent to a diploid dosage in the female relative. The presence of a band with double intensity provided a means of comparison with the putative deleted band suspected of having haploid dosage and calculating a ratio between the band intensities on the autoradiogram. To assess relative dosage, the band ratios between the female being evaluated and a normal, nondeleted control run on the same blot were compared. Band ratios were used rather than single band intensities to account for differences in DNA transfer from lane to lane.

The band intensities on autoradiograms were scanned using the Helena Electrophoresis Data Center (EDC) which is

the scanning densitometer component of the Helena Rapid Electrophoresis Analyzer (RPA, Helena Laboratories, Beaumont, Texas). This instrument is routinely used in the Clinical Chemistry laboratory for CK and lactic dehydrogenase (LD) isoenzyme quantitation and has the flexibility to provide many other functions as indicated by the present application. The Helena EDC is a multipurpose densitometer coupled to a computer, display and printer. The presence of a hard disc allows all data to be stored and recalled for later use. The instrument was set at a wavelength of 525 nm and a slit size of 5 (4 mm length by 0.4 mm width). Each film was scanned and the band intensities for each lane (representing 1 DNA sample) were calculated and provided on individual printouts for each sample. Using the versatile program capabilities of the Helena EDC, the user can easily perform baseline setting and editing functions with printouts available prior to and at completion of the editing procedure. Careful alignment of the autoradiogram film in the densitometer platform and programming of the specific lanes to be scanned were important to ensure that accurate scanning of each lane occurred.

For densitometry precision studies the autoradiogram was removed and repositioned between each replicate scan and the EDC was reprogrammed to reset the scan parameters.

In all densitometry analyses, with the exception of the linearity studies, values for each fraction were calculated as the relative percent of the total. In the linearity

studies, the value of each fraction was calculated as an integral by using an internal standard as a reference set to a value of 100. The internal standard was prepared by applying a piece of black, opaque tape approximately the size of a band in one lane of the autoradiogram. The internal standard was scanned and edited prior to scanning the sample. Manual gain and manual zero modes on the EDC were used since the background on each autoradiogram varied considerably with increased exposure.

Creatine Kinase

Serum creatine kinase measurements were performed by the Clinical Chemistry laboratory, Medical College of Virginia, Richmond, Virginia, on the Cobas-Bio centrifugal analyzer (Roche Analytical Instruments).

Results and Discussion

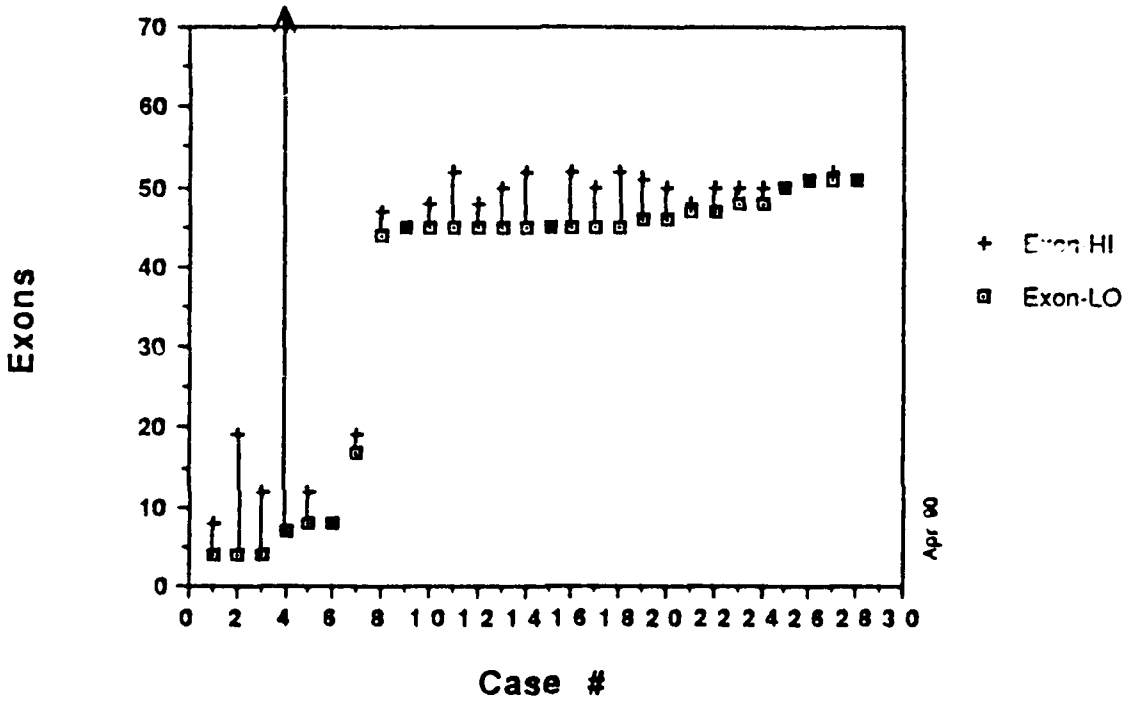
Screening for Deletions by PCR

Introduction

Using the multiplex PCR method 65 unrelated males affected with X-linked muscular dystrophy have been screened for the presence of deletions in the dystrophin gene. Deletions have been identified in 28 patients which represents 43\5 of the population tested. There were 24 DMD and 4 BMD patients among those deleted. Eighty-six percent of the deletions (24/28) were found using a multiplex of 6 PCR primers (a-f), while 4 deletions were seen with the incorporation of 3 additional primers (g, h, i) to yield a multiplex of 9 primers. The addition of primers g, h and i also extended 3 previously observed deletions found with primers a-f. The location of these deletions in reference to the exons of the dystrophin gene is shown in Figure 3.

The first section of the results will provide examples of deletions in the dystrophin gene observed in males affected with X-linked muscular dystrophy by PCR multiplex analysis. The presence of a deletion is indicated by the failure of a primer set to anneal and therefore amplify at

Figure 3. **Deletion Patterns in Patients with BMD and DMD**



specific sites. This results in the absence of bands for PCR reaction products corresponding to a specific primer set.

Initially there is a discussion of optimization of reaction conditions using single primer sets and multiple primer sets. A presentation will follow of patient results demonstrating deletions in different gene locations obtained with multiplex PCR analysis using either the 6-plex or the 9-plex screening method.

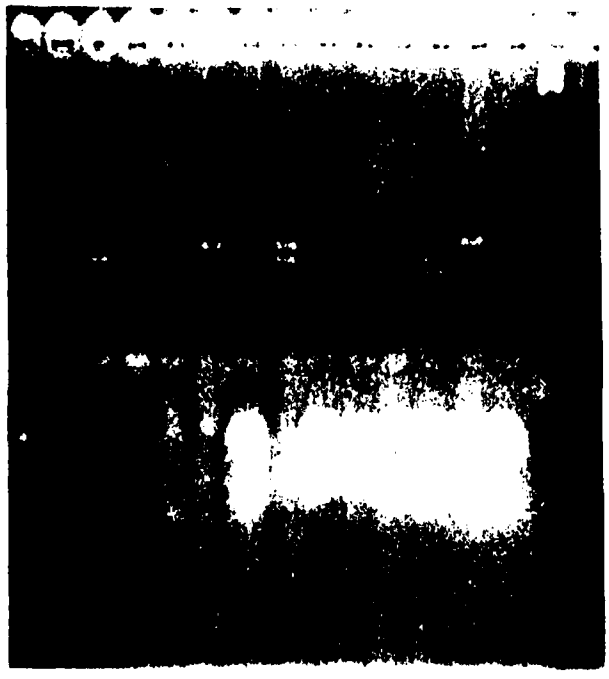
Identification of the sites of deletions in affected males allowed selection of appropriate cDNA probes to evaluate female relatives for deletion in the same gene region. Determination of gene dosage for either haploid or diploid status was made by comparing autoradiogram band intensities of a potential deleted restriction fragment to a nondeleted fragment between suspected carriers and normal controls. Five family studies are discussed which represent both obligate and simplex cases to demonstrate the use of band intensity ratios in determining the probability of carrier status. Sequences of the oligonucleotide primers used in the multiplex reaction with their location in the gene are shown in Table 1, Materials and Methods.

Multiplex Reaction Optimization

Reaction conditions were established using modifications of conditions published by Chamberlain (116)

and Kogan (113). Experiments were performed initially to establish optimum concentrations of magnesium in the 10 X reaction buffer, Taq polymerase, primers, template DNA and agarose. Additional parameters varied were the electrophoresis voltage, ionic strength of electrophoresis buffer and time and temperature of amplification cycles. It should be emphasized that addition of each new set of primers may require modification of reaction conditions. Different combinations of primers seem to require different conditions for optimum performance and as new primers are added there is less flexibility in permissive reaction conditions. In preliminary studies, using each primer independently to amplify nondeleted control DNA, 6 distinct bands (a-f) were observed after electrophoresis on agarose gels (Figure 4). As additional primers (g, h and i) were available, similar experiments were performed (Figure 4). No extraneous bands were observed. While one set of primers may be specific when used separately, the presence of additional primer sets may lead to spurious amplification. In assessing specificity it is also useful to include a patient DNA with a known deletion. Table 2 shows the size of PCR products obtained with each primer and the order of migration in agarose gels. It also provides the relative position of the amplified products when primers were used in a 6-plex reaction (a-f), in 2 separate sets of 3-plex reactions (f, c, d) and (e, b, a) and as a 9-plex composed of a 5-plex (e, b, a, g, i) and a 4-plex (f, c, h, d).

Figure 4. PCR using Individual Primers



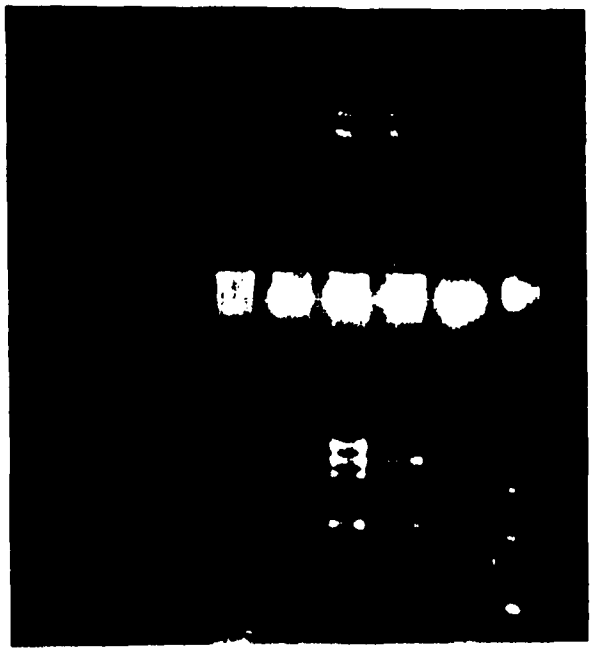


Table 2

PCR Multiplex Amplification Products: Gel Migration Pattern by Size

6 Primers	6 Primers	9 Primers
as 6-plex	as 3-plex A[2]	as 5-plex[3]
e (547)[1]	f (506)	e (547)
f (506)	c (459)	b (416)
c (459)	d (268)	a (360)
b (416)	3-plex B[2]	g (331)
a (360)	e (547)	i (196)
d (268)	b (416)	4-plex[3]
	a (360)	f (506)
		c (459)
		h (409)
		d (268)

[1]Amplification size in base pairs.

[2]3-plex A is located at the top and 3-plex B at the bottom of each lane in subsequent figures of PCR results.

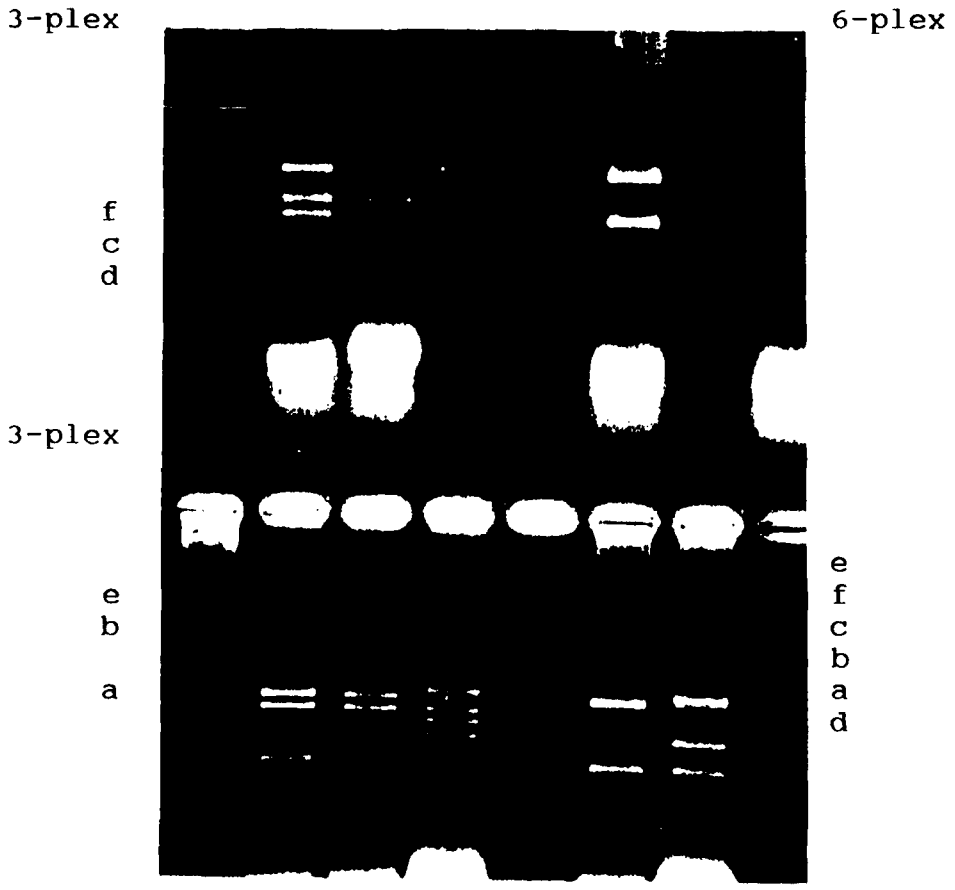
[3]The 5-plex is located at the top and the 4-plex at the bottom of each lane in subsequent figures of PCR results.

Experiments were performed with a set of 6 primers in one multiplex reaction or separated into two 3-plex reactions for a nondeleted control DNA and patient DNA with a known deletion. Not all bands were well resolved with the 6 primer multiplex, while 2 separate 3-plex reactions resulted

in much better resolution and stronger band intensities. Figure 5 provides a comparison of results for the nondeleted control using two 3-plex reactions (lane 2: top with primers f, c and d, bottom with primers e, b and a) or a single multiplex reaction containing all 6 primers (lane 4: bottom with primers a-f). In the 6-plex all bands are resolved except the 2 largest bands, e and f. Results for the deleted control are shown in lane 6 with the top 3-plex showing a deletion of the band for primer b and the bottom 3-plex showing a deletion of the band for primer c. The lower position of lane 7 presents results of the 6-plex screen of the patient DNA in which the deletion of bands c and b is seen; however, there is poor resolution between the 2 largest bands e and f. These results also illustrate another problem with amplification by primer f which consistently produced a band of weaker intensity in a 6-plex reaction. When used as part of a 3-plex (f, c, d) primer f amplified just as well as the other primers. This finding has been reported by others and appears to be a common occurrence (104).

To circumvent the problems of decreased band intensity with primer f and to improve resolution we routinely used two separate 3-plex reactions for screening. While this approach necessitates setting up 1 more reaction, it offers the advantage of easier interpretation and fewer repeats.

Figure 5. PCR using Two 3-plex Reactions or a 6-plex Reaction

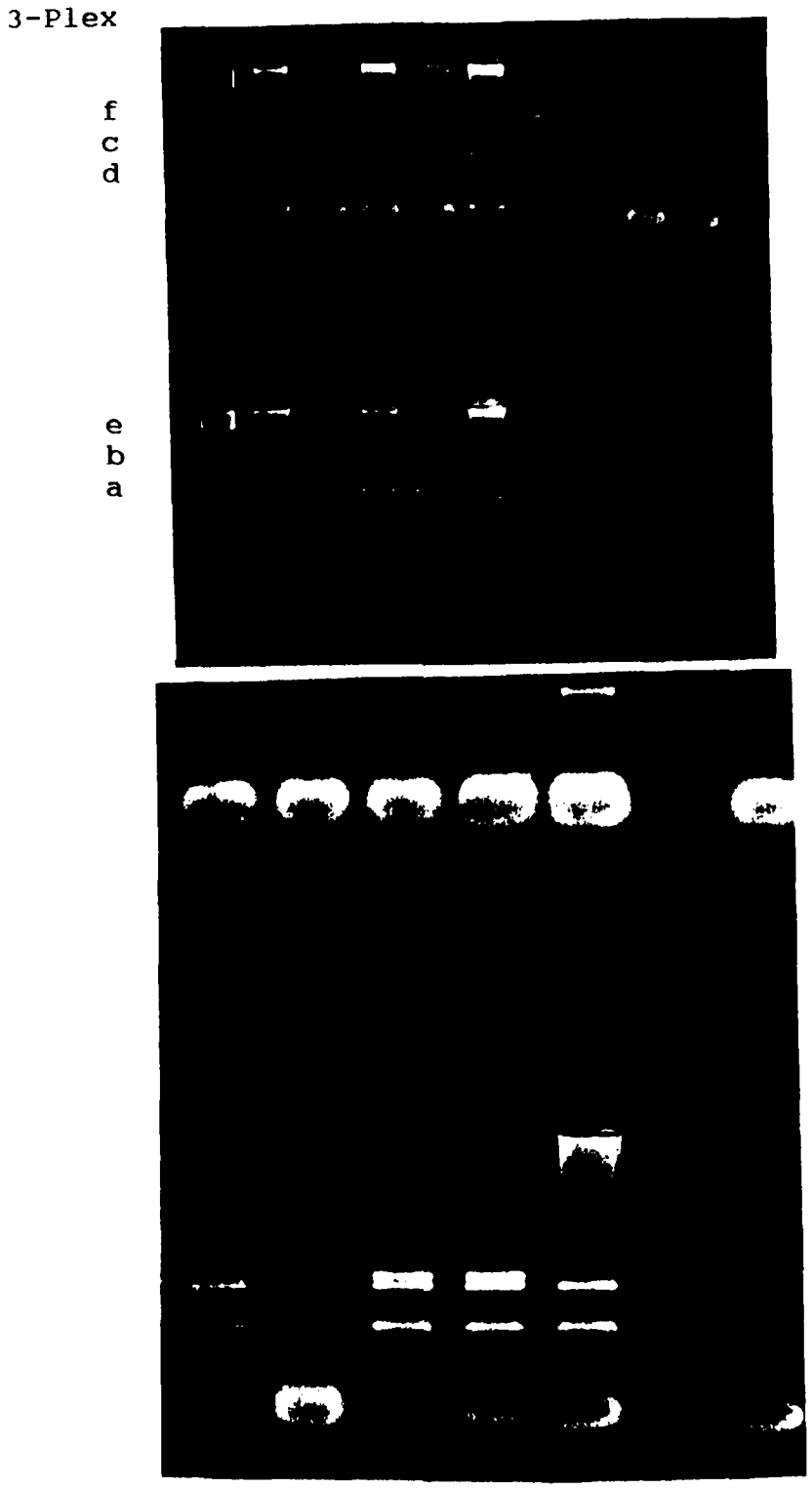


Results of Patient Screening by Multiplex PCR

All patients were initially screened using two separate 3-plex reactions (f, c, d and e, b, a). Figure 6 shows representative examples of the deletions detected. In panel A, a deletion of band f (top 3-plex) and band e (bottom 3-plex) was observed in patient D. W. (lane 2) and P. W. (lane 6), while patient J. L. in lane 3 had a deletion of band c (top 3-plex) and bands b and a (bottom 3-plex). An enlarged view of the same gel is shown in panel B which more clearly demonstrates the deletion of these bands. Target sequences for primers e and f are located in the region of cDNA 7 and 8, while those for primers a, b and c are situated in the areas of probes 1 and 2. In lane 1 the 123 base pair marker provides a measure of relative migration and the nondeleted control in lane 9 demonstrates the 6 anticipated bands. The absence of bands in the reagent control in lane 10 indicates that the PCR reagents are free of contamination by exogenous DNA.

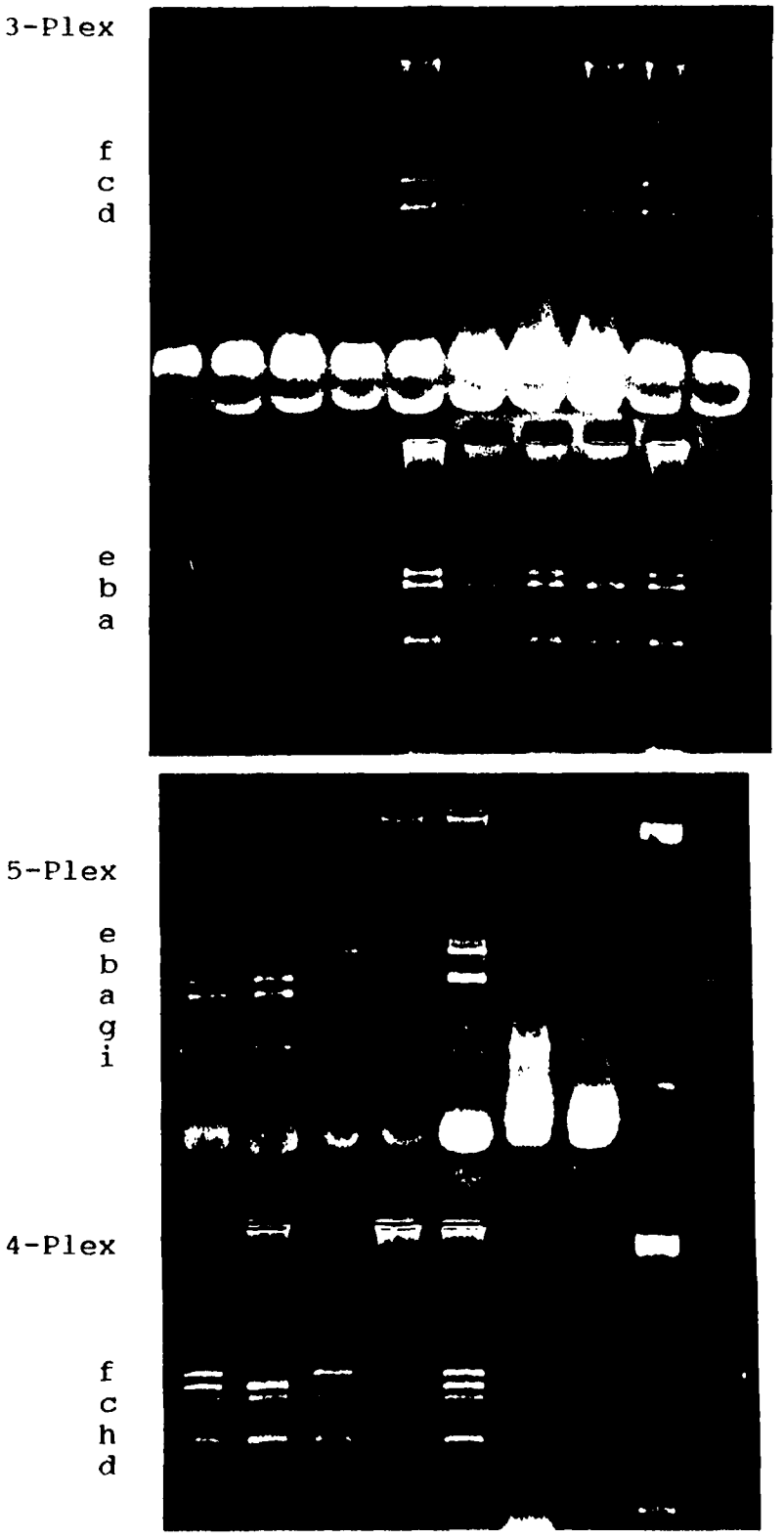
Panel A in Figure 7 provides additional examples of deletions identified. In lane 2, patient E. C. shows a deletion of band a (bottom 3-plex), patient R. C. a deletion of band f (top 3-plex) and e (bottom 3-plex), and patient S. M. the deletion of band f (top 3-plex). In patient S. M. there had been some difficulty in making a differential diagnosis between DMD and polymyositis; the finding of a deletion provided clarification in this case in favor of DMD.

Figure 6. Deletions Detected by Multiplex PCR in MD Patients



In Figure 7, panel B, deletion patterns are shown for the 9 primer multiplex as the combination of a 4-plex and a 5-plex. In lane 1, a nondeleted control exhibits amplification of all bands (top is 5-plex: primers e, b, a, g, i and bottom is 4-plex: primers f, c, h, d). A deletion of band e (top 5-plex) and band f (bottom 4-plex) is seen in patient J. W. in lane 2, and a deletion of bands b, a, g and i (top 5-plex) and band c (bottom 4-plex) is observed in patient J. L. in lane 3. J. L. was previously shown to have a deletion of bands a, b and c by the 6-plex and the additional primers extended the deletion by 3 exons in the 5 prime direction. In lane 4 the absence of all bands indicates a large deletion in patient T. M., who has multiple disorders including DMD, glycerol kinase deficiency and adrenal hypoplasia. There have been a number of reports of patients with this constellation of diseases who demonstrate large deletions in the Xp21 and Xp22 regions to include portions of the DMD gene and extend teleomerically (3 prime) into the L1-4 locus. The L1-4 locus is believed to contain genes for glycerol kinase deficiency and adrenal hypoplasia (121-124). In such cases it would be useful to have an additional set of primers derived from another location in the genome to provide a positive control for amplification. The deletion should also be confirmed by Southern analysis with cDNA probes.

Figure 7. Deletions Detected by Multiplex PCR in MD Patients



Deletion Frequency

While there is a heterogeneous distribution of deletions the majority are found in the regions of cDNA probes 7 and 8. Table 3 provides a summary of the relative frequency of deletions detected by the 9 primer multiplex method. The relative frequencies seen in the present population (65 patients) is compared to those reported by Chamberlain et al (104, 116) in a study of 200 patients. the present study exhibits a higher percentage detected in the area of cDNA probes 7 and 8 than in the previous study, which may be a reflection of the smaller sample size. Since the present set of primers are directed only to the areas of probes 7 and 8 and 1-3 and detect deletions in 43% of patients, it would be expected that additional primers covering other regions of the gene should detect deletions in another 5 to 10% of patients. Additional deletions will also be identified with cDNA probes which are reported to detect deletions in 50 to 60% of males with muscular dystrophy.

In the present study, deletions were found by the multiplex PCR in 2 males who were previously shown to be deleted in the region of cDNA probes 2-3 by RFLP analysis with pERT 87-8 (93, 125). Further evidence for the validity of the PCR multiplex method has been the confirmation of deletions detected by PCR in all males in the present study who have been evaluated by Southern analysis with cDNA probes (22/27, 79%).

Table 3

Relative Frequency of Deletions in Dystrophin Gene by PCR Multiplex Screen

Primer	cDNA Probe/Exon	% Deleted Present Study	% Deleted Literature Results
i	1/4	6 (4/65)	6
a	16/8	8 (5/65)	11
g	26/12	5 (3/65)	10
b	3/17	5 (3/65)	9
c	3/19	5 (3/65)	10
d	7/44	3 (2/65)	4
e	7/45	20 (13/65)	8
f	8/48	23 (15/65)	18
h	8/51	11 (7/65)	13

Note: Present study screened 65 patients; literature results in 200 patients (references 109, 119).

At this time females cannot be screened for deletions by PCR since the normal X chromosome is amplified, which prevents detection of heterozygosity at the DMD locus.

The PCR multiplex protocol provides a rapid and comprehensive method of simultaneously screening large populations of affected males for deletions at 9 locations in the dystrophin gene. The entire protocol can be performed in 1 to 2 days compared to cDNA probe hybridization analysis which in our laboratory requires 2 to

3 weeks for each probe. The multiplex PCR method has the added advantage of being technically straightforward and not requiring radioisotope.

DMD Carrier Detection by cDNA Probes

Introduction

The identification of deletions in 43% of affected males provided the information needed to select appropriate cDNA probes to evaluate female relatives for carrier status. In the course of this investigation, 37 females representing 21 unrelated kindreds have been evaluated by cDNA probes to determine gene dosage by quantitating restriction fragment intensities with a densitometer.

Table 4 provides a summary of the families in which carrier status could be determined. Determinations have been possible in 24 of 37 females representing 65% with 14 low probability carriers (LPC) and 10 high probability carriers (HPC) identified.

In the remaining 13 females, carrier determination has not as yet been possible. In patient T. M., shown in Figure 7, panel B, the presence of a deletion spanning a large area of the gene was indicated by the absence of all 9 bands in PCR multiplex analysis. By cDNA analysis his DNA also fails to hybridize with any of the probes tested to date (126) which has prevented cDNA analysis of his mother, since quantitation of gene dosage requires the presence of at least one restriction fragment band to calculate a ratio

Table 4

DMD/BMD Carrier Determination by Densitometry

Name	Exon Deleted		cDNA Probe 8 Restriction Fragment						
	Low	High	10kb	7kb	3.7, 3.8kb	3.1kb	Ratio	Con-ratio	Status
J.B. DMD male	51	51				del			
M.B. mother					x	0	1.94	0.92	HPC
J.B. sister					x	0	0.92	0.92	LPC
P.B. sister					x	0	1.86	0.92	HPC
D.C. BMD male	45	48	del		del 3.8				
B.C. mother			0	x			0.48	0.52	
					0	x	0.89	0.74	LPC
K.C. sister			0	x			0.44		
					0	x	0.85		LPC
C.D. BMD male	51	54*		del		del			
B.D. mother			x	0			0.46	0.47	
					x	0	0.83	0.95	LPC
D.D. BMD male	50	50			del 3.7				
C.D. mother									Oblig
J.H. aunt					0	x	1.05	0.92	LPC

Table 4 (cont'd.)

DMD/BMD Carrier Determination by Densitometry

Name	Exon Deleted		cDNA Probe 8 Restriction Fragment						
	Low	High	10kb	7kb	3.7, 3.8kb	3.1kb	Ratio	Con-ratio	Status
J.F. DMD male	46	51	del		del	del			
E.F. mother				x	0		2.69	1.84	HPC
				x		0	2.26	1.38	
L.F. sister				x	0		2.69		
				x		0	2.23		HPC
B.F. sister				x	0		4.55		
				x		0	2.23		HPC
S.F. sister				x	0		1.80		
				x		0	1.58		LPC
P.K. BMD male	45	48	del		del 3.8				
E.K. mother					0	x	0.96	0.89	LPC
S.K. DMD male	48	50			del				
L.K. mother					0	x	0.80	0.67	LPC
A.L. DMD male	45	50	del		del				
E.L. mother			0	x			0.50	0.48	LPC
					0	x	0.67	0.70	

Table 4 (cont'd.)

DMD/BMD Carrier Determination by Densitometry

Name	Exon Deleted		cDNA Probe 8 Restriction Fragment						
	Low	High	10kb	7kb	3.7, 3.8kb	3.1kb	Ratio	Con-ratio	Status
M.M. DMD male	46	48	del		del 3.8				
M.M. mother			0	x			0.18	0.52	Oblig
					0	x	0.65	0.74	
S.M. DMD male	46	50	del		del				
J.M. mother					0	x	0.86	0.7	LPC
C.R. sister					0	x	0.96	0.7	LPC
J.S. DMD male	47	50	del		del				
G.S. mother			0	x			0.22	0.41	
					0	x	0.37	0.67	HPC
P.S. cousin			0	x			0.70	0.64	
					0	x	0.71	0.79	LPC
A.S. DMD male	48	50	del		del				
S.S. mother					0	x	0.51	0.67	HPC

Table 4 (cont'd.)

DMD/BMD Carrier Determination by Densitometry

Name	Exon Deleted		cDNA Probe 8 Restriction Fragment						
	Low	High	10kb	7kb	3.7, 3.8kb	3.1kb	Ratio	Con-ratio	Status
M.S. DMD male	44*	47	x						
R.D. mother			0	x			0.26	0.63	HPC
J.S. sister			0	x			0.60	0.63	LPC
J.W. DMD male	45	50	del		del				
T.W. mother			0	x			0.43	0.52	
					0	x	0.83	0.74	LPC
J.W. sister			0	x			0.45	0.52	
					0	x	0.88	0.74	LPC

Ratios calculated for restriction fragment intensities in relative percent of total for 10/7, 3.7,3.8/3.1, 7/3.7,3.8, 7/3.1.

Conratio represents ratio for control DNA.

For each female (x) represents fragment not deleted, diploid dosage and (0) represents fragment deleted in male, possible haploid dosage in female.

Asterisk (*) indicates that deletion is not fully mapped.

HPC represents high probability carrier.

LPC represents low probability carrier.

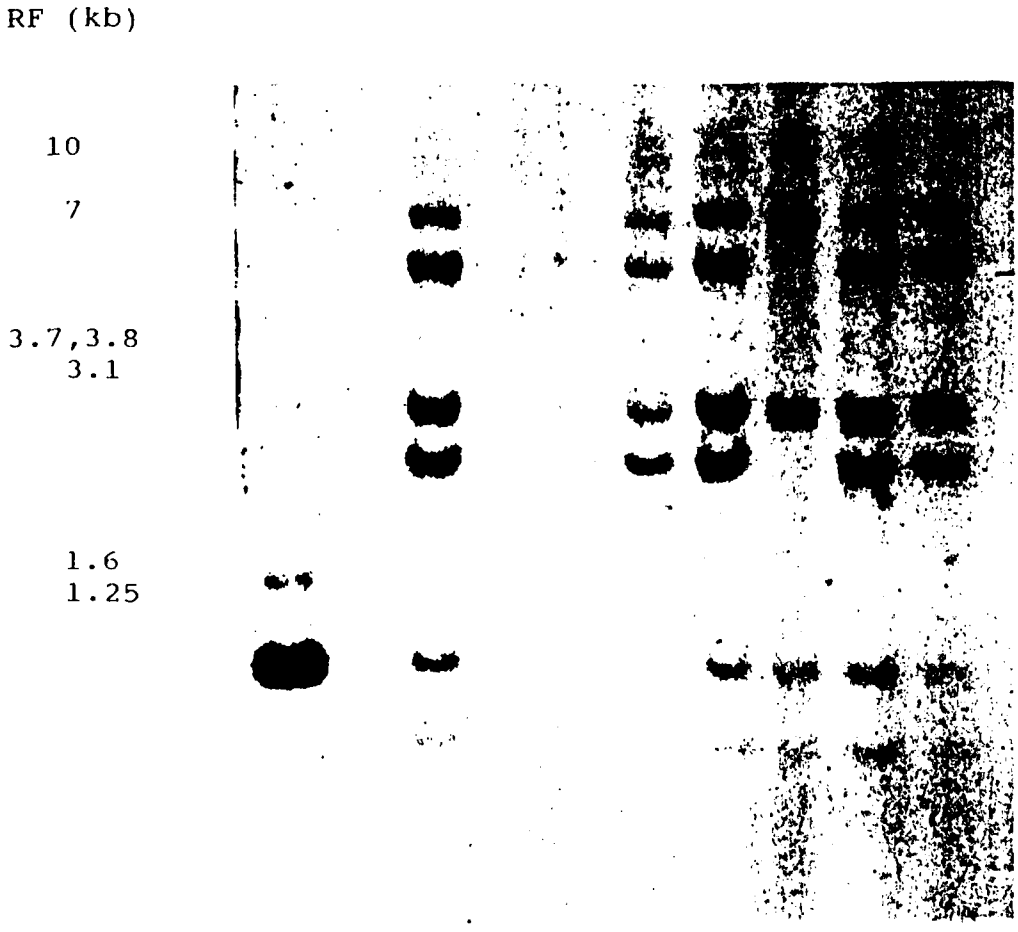
with a deleted band. In the present study we have limited ourselves to those cases that involve probe 8. There are 8 females who cannot be evaluated by probe 8 since the affected male is either deleted for the entire region of

probe 8 or is not deleted for any bands in this area. The autoradiogram in Figure 8 contains 3 cases in which hybridization with probe 8 was not useful due to the type of deletion present. Patient P. W. in lane 2 and M. L. in lane 4 had deletions of all 6 HindIII restriction fragments identified with probe 8, while patient D. B. in lane 5 showed no deletion with this probe. Since deletions were detected in all 3 males by PCR primers e and f, the general strategy would be to first hybridize with probe 8 since it has provided the most efficient approach in hybridization studies.

While the previously described filters have been rehybridized with probe 7 in which there is a deleted and a nondeleted band, allowing the calculation of dosage ratios, there is currently insufficient data for normal control ratios for this probe to make an interpretation in female relatives. In the remaining 4 females there were problems with the restriction digest or DNA transfer which prevented analysis.

While 13 of the 65 families were considered to be obligate cases, only 2 of these males were found to have deletions by PCR and 1 additional deletion was identified by cDNA analysis. The availability of obligate females was especially valuable in establishing the validity of quantitation of gene dosage for carrier assessment.

Figure 8. Autoradiogram 90-4, Hybridization with DMD cDNA Probe 8

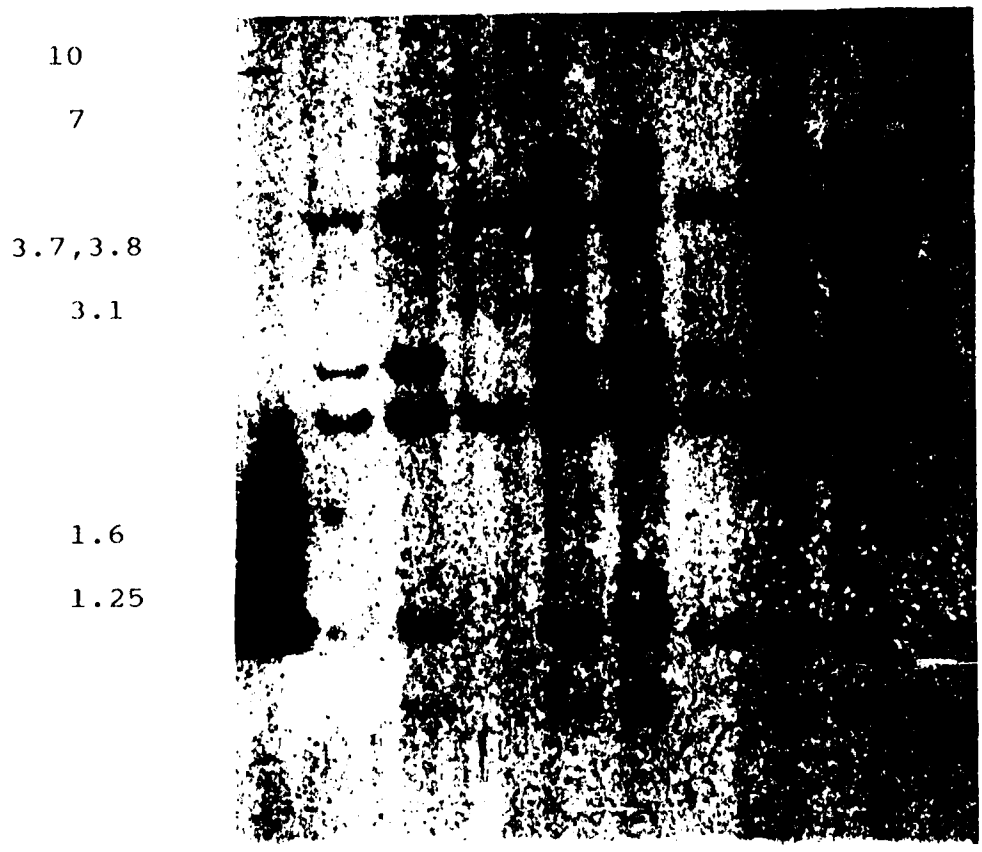


Dosage Ratios by Densitometry

Several family studies will be described to illustrate the use of dosage ratios quantitated by densitometry to make carrier determinations. The 6 HindIII restriction fragments detected by cDNA probe 8 are identified adjacent to the figures of autoradiograms which follow.

Family 1. In family 1 the mother is an obligate carrier having 2 affected sons. The male, M. M., had a deletion in the area of band f by multiplex PCR. The autoradiogram in Figure 9 shows the affected male in lane 1 to be deleted for the 10, 3.8 and 1.25 kb bands. In the mother, shown in lane 2, the band intensities were measured and ratios calculated for the 10 to 7 kb bands providing the ratio of a deleted to a nondeleted band. If the mother has the deletion then one would expect to find band 10 kb present in haploid dosage as compared to diploid dosage for the 7 kb band. As shown in Figure 10 the densitometric scan revealed a 10/7 ratio of 0.22 in the mother, M. M. The same band ratio was determined for a nondeleted control from the same blot which showed a value of 0.54. The mother appears to have approximately half the dosage observed in the control for the 10 kb band which is the same deletion found in her son. As observed by others (103) the 10, 7, 3.7/3.8 and 3.1 kb bands demonstrate the strongest intensities and are therefore the most useful in dosage calculations.

Figure 9. Autoradiogram 90-1, Hybridization with DMD cDNA Probe 8



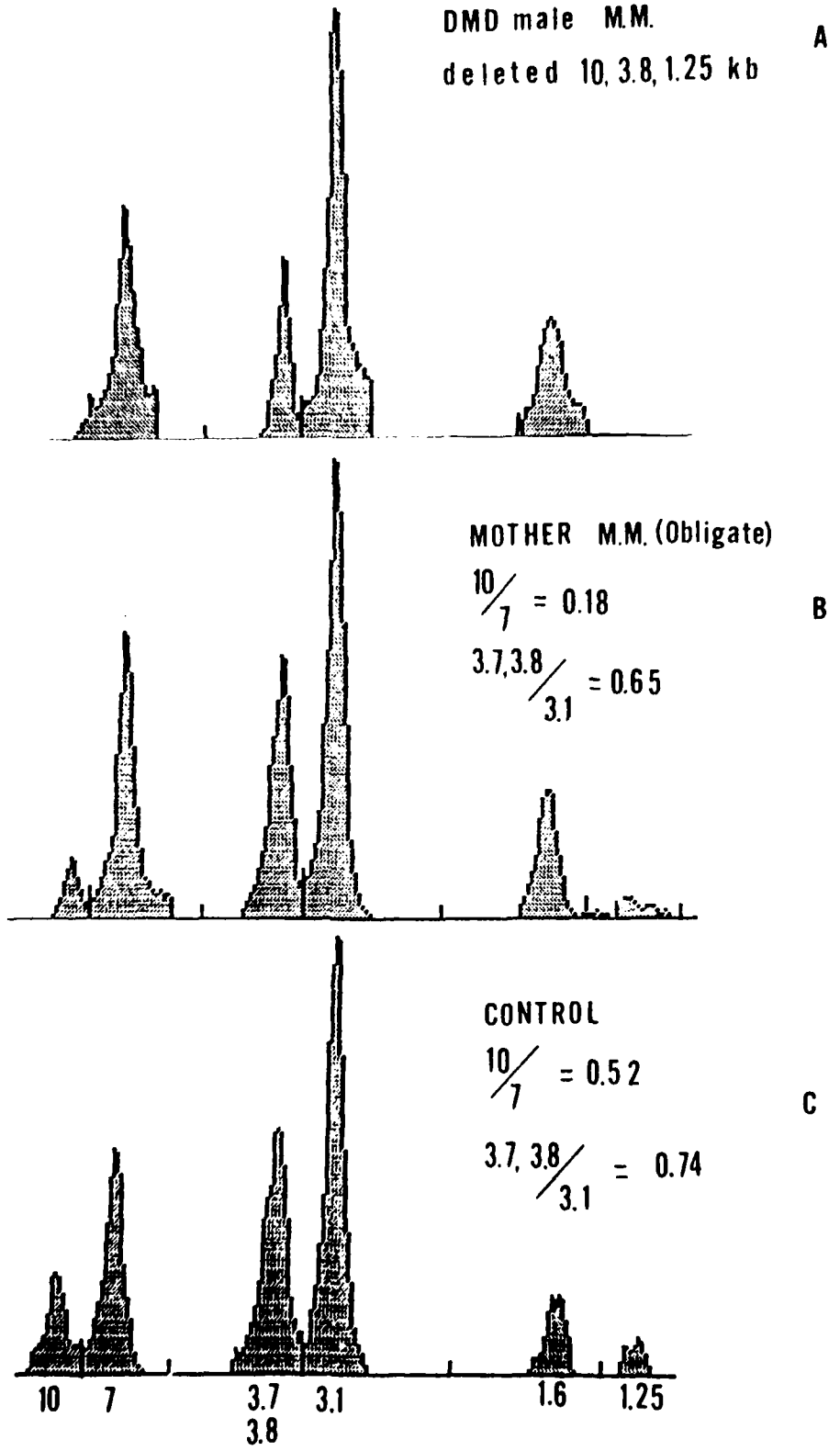


Figure 10. Family 1 densitometric scan

While interpretation of carrier status is facilitated by having more than one set of ratios, it is not always possible. In family 1, M. M is deleted for only the 3.8 kb, so the ratio of 3.7,3.8/3.1 in his mother, M. M., while showing a slight reduction in dosage intensity, still falls within the lower end of the reference range established for this band ratio (0.59 to 1.03, mean = 0.81). Methods for determination of reference ranges are discussed in a subsequent section, Reference Intervals for Dosage Ratios.

Family 2. Hybridization results with cDNA probe 8 are shown in Figure 9 for family 2. The male, D.C. has a diagnosis of Becker muscular dystrophy and was previously shown to be deleted in the regions of primers e and f by multiplex PCR. As shown in lane 7, D.C.'s DNA fails to hybridize with the 10, 3.8,1.6 and 1.25 kb fragments which confirms the PCR screen results. His mother (B. C.) and sister (K. C.) are shown in Figure 9, lanes 8 and 9, while the nondeleted control is in lane 10. Dosage ratios were calculated for the bands 10/7 and 3.7,3.8/3.1. As shown in the densitometric scans in Figure 11 (B) the mother (B. C.) demonstrates values of 0.48 (10/7) and 0.88 (3.7,3.8/3.1) while the sister (K. C.) Figure 11 (C) has values of 0.44 and 0.86. When compared to the control ratio in Figure 11 (D) of 0.52 (10/7), both females appear to have diploid dosage for the 10 kb fragment deleted in the male and therefore have reduced probability of being carriers. This case is another example of the limited usefulness of the

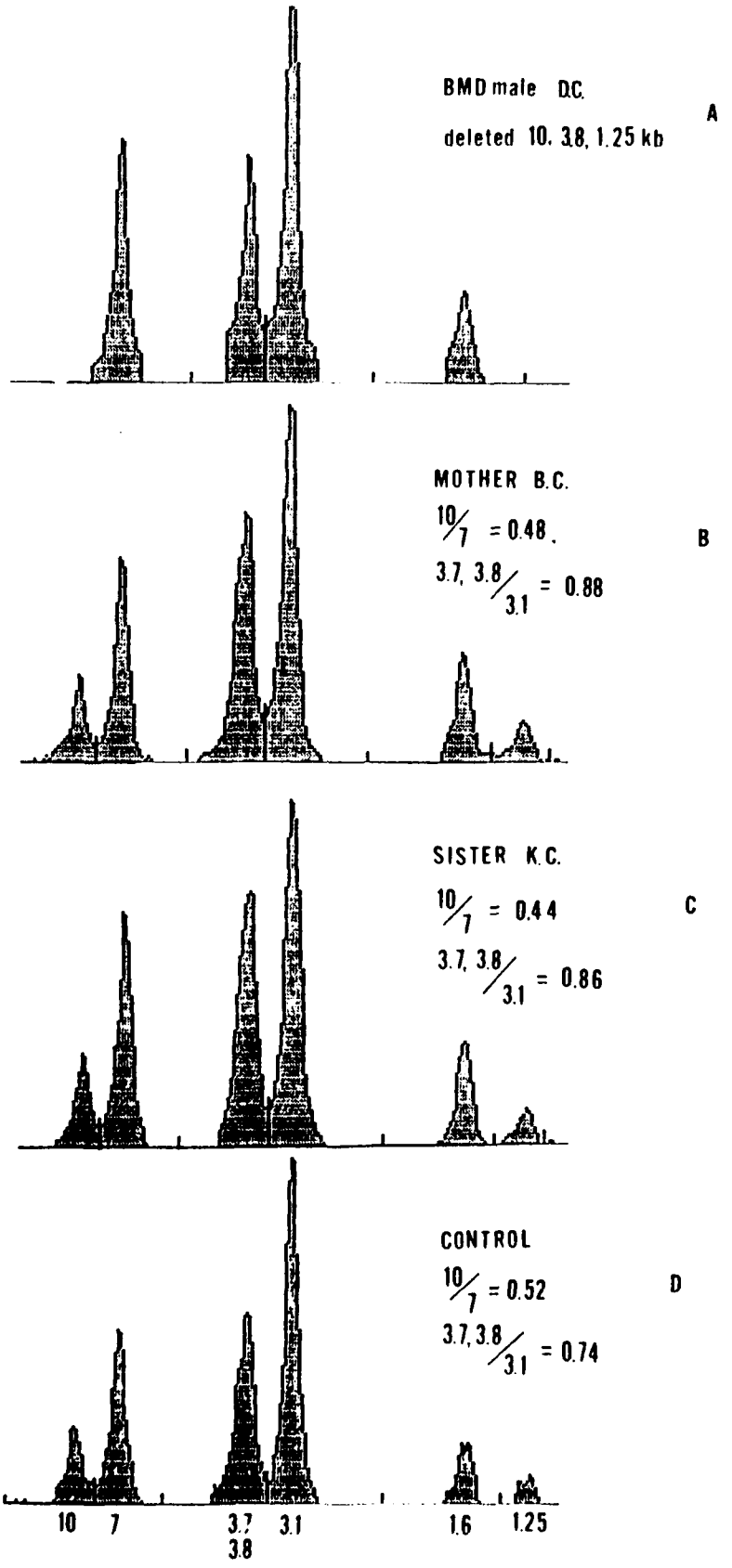


Figure 11. Family 2, densitometric scan

3.7,3.8/3.1 ratio when only the 3.8 kb fragment is deleted in the male; however, in this case the mother and sister, both determined to be low probability carriers, demonstrate values comparable to the control and close to the mean for the reference range (mean = 0.81). In family 1 the mother, M. M., an obligate carrier who should be deleted for the 3.8 kb fragment, had a somewhat lower value (0.65) for this ratio.

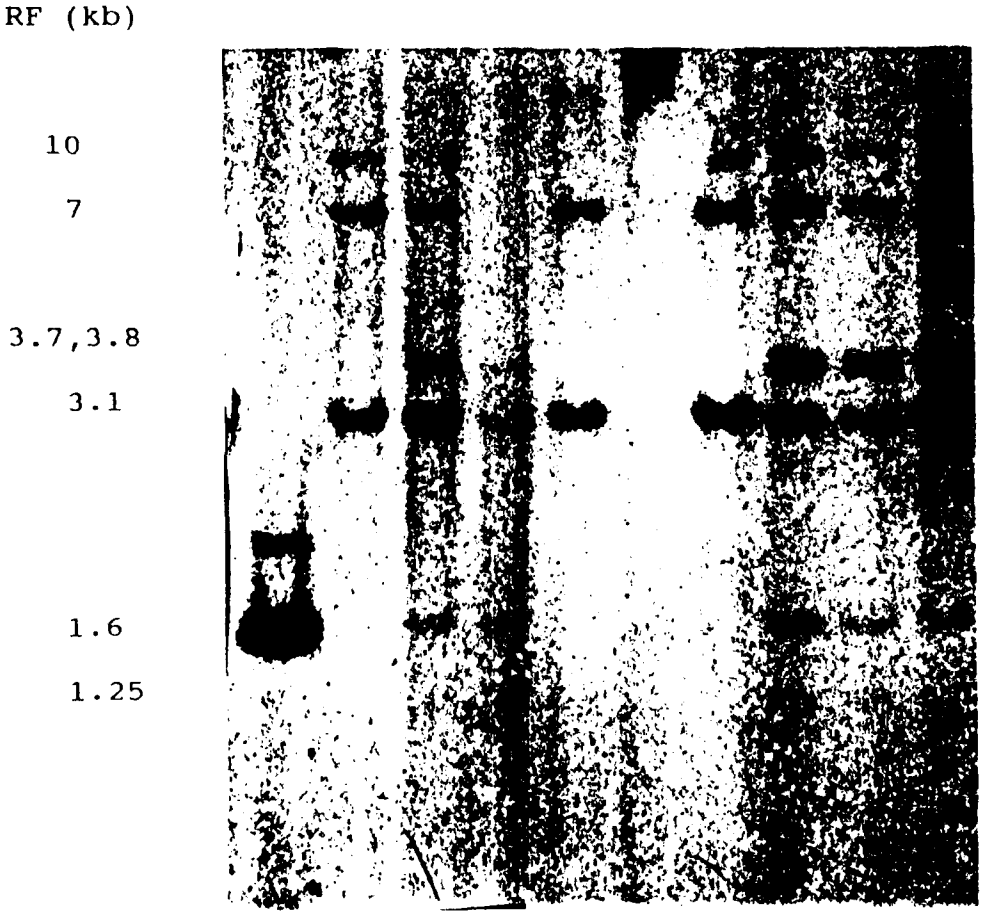
It is of interest with regard to phenotypic variation that D. C. and P. K., BMD males, are deleted for similar regions of probe 7 and 8 as J. W., a DMD male shown in lane 4, Figure 9. While they all have a deletion breakpoint between the 4.1 and 0.5 kb fragments in probe 7 (data not shown), the deletion breakpoint in probe 8 is different. J.W. is deleted for 2 additional fragments 1.6 and 3.7 kb which lie more 3′ in the gene. Analysis of the border type for intron/exons in this region suggests that D. C. and P. K. deletions might leave the translational reading frame intact while J.W.'s deletion might disrupt the reading frame which may account for the difference in disease severity observed in these 2 males.

Exon borders are designated as to the relative position of each intron/exon border with respect to the translational reading frame. A border of type 3 indicates that the intron/exon border occurs between intact codons in the mRNA, after the codon in position 3. A border of types 1 cr 2 indicates that the border occurs after the first or second

nucleotide of the codon respectively (75). If a deletion juxtaposes the 3′ exon border of the exon preceding the deletion to a 5′ exon border of the same type, the translational reading frame is maintained. If the deletion juxtaposes differing 3′ and 5′ border types, a frameshift occurs. In the BMD patients, D. C. and P. K., the deletion is predicted to bring together exons of the same border type and would be expected to maintain an open reading frame. Such a deletion would result in a protein product with only a small internal deletion. In J. W. the deletion is predicted to bring together a 3′ exon border of type 3 with a 5′ exon of type 1 which would be predicted to shift the reading frame. Such a deletion would result in a truncated protein deleted for approximately 40% of the carboxyl terminal region. Direct evidence for a translational frameshift could only be provided by sequencing the deletion breakpoints.

Family 3. In family 3 hybridization studies revealed the same deletion in the mother as found in her son. The DMD male, J. S., had a deletion in the area of primer f by the multiplex PCR screen. This deletion was confirmed by hybridization with probe 8 which identified an omission of the 10, 3.7, 3.8, 1.6 and 1.25 kb fragments as shown in Figure 12, lane 5. The mother (G.S) is shown in Figure 12, lane 10, and the control in lane 9. Densitometric scans of the autoradiogram are shown in Figure 13 (B) in which G. S. demonstrates a dosage ratio of 0.22 (10/7) and 0.5

Figure 12. Autoradiogram 90-2, Hybridization with DMD cDNA Probe 8



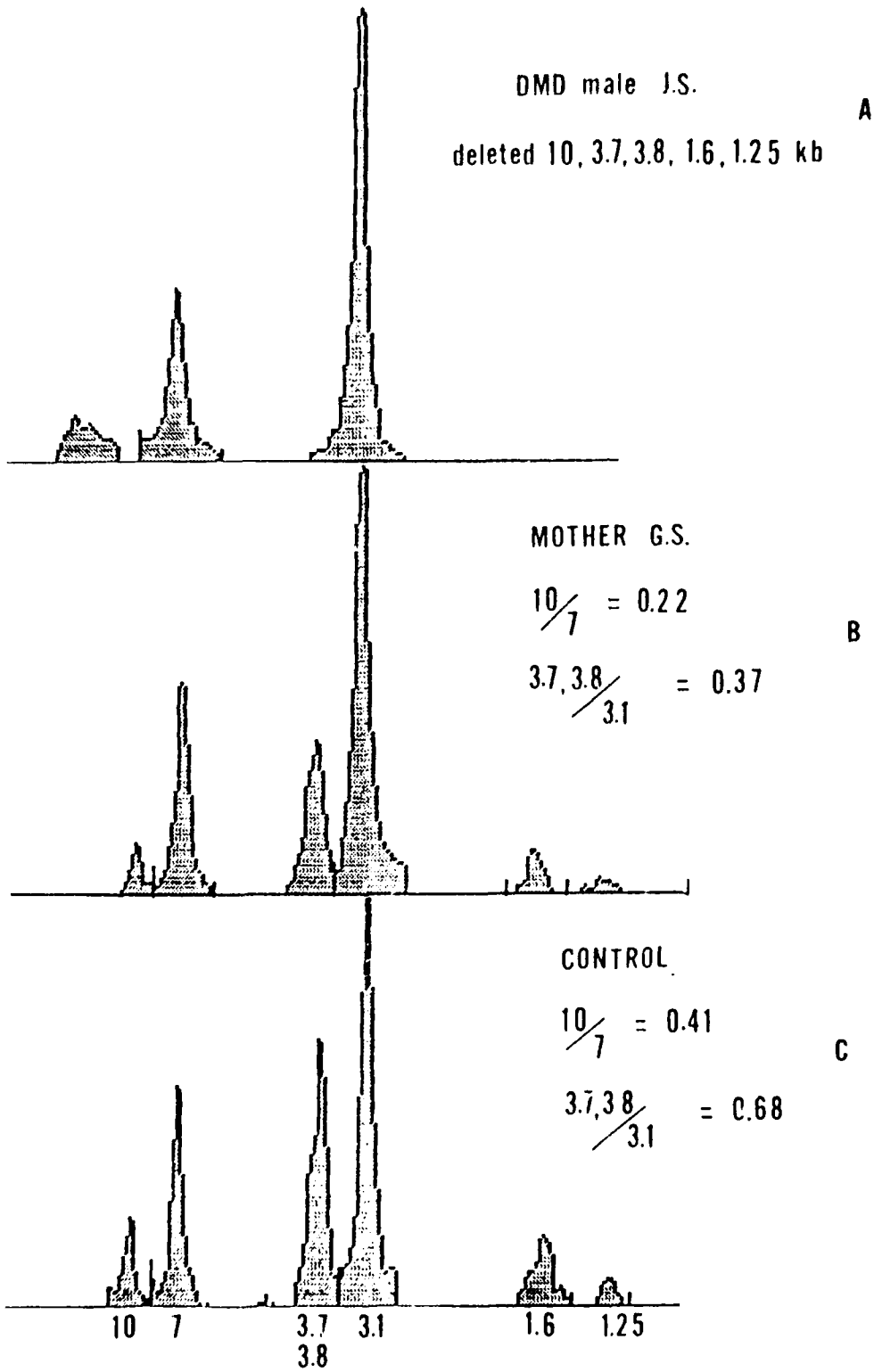
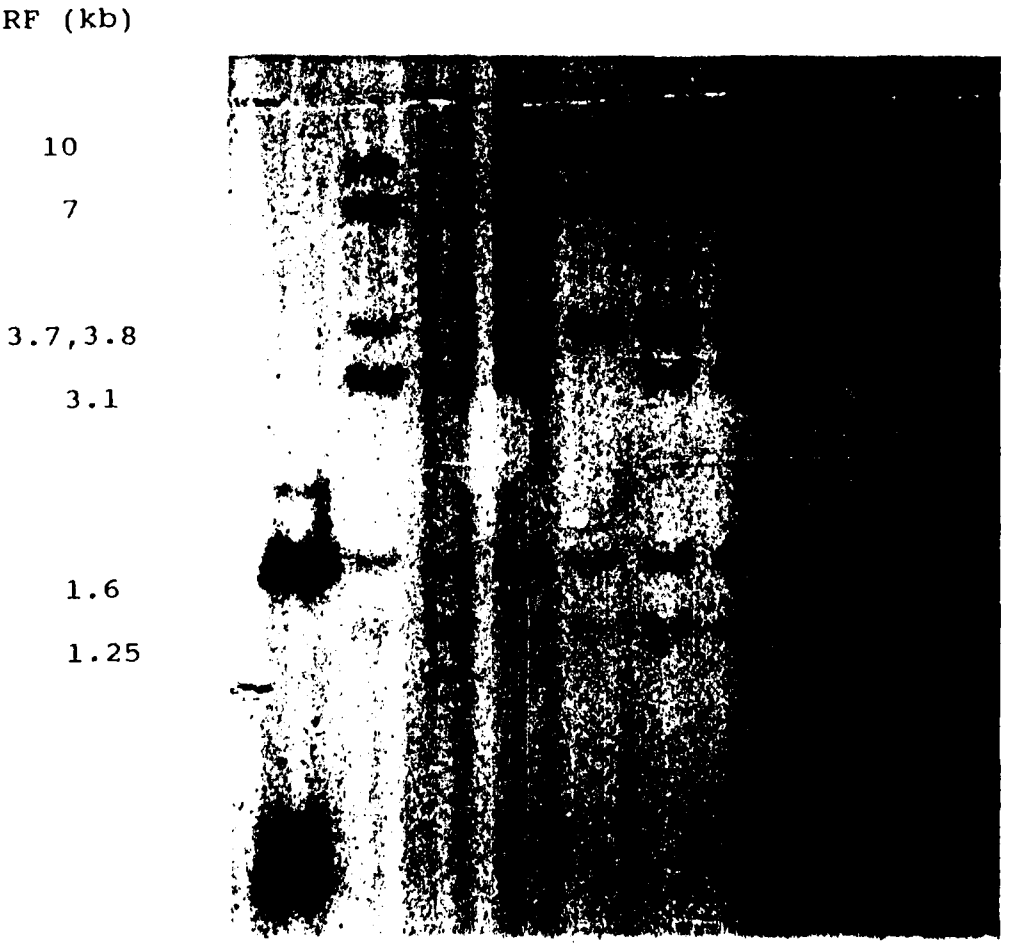


Figure 13. Family 3, densitometric scan

(3.7,3.8/3.1), while the control has ratios of 0.50 and 0.77. The data indicates a haploid dosage of both the 10 and the 3.7,3.8 kb fragments in G. S. as seen in the reduction in band intensities by approximately 50 percent which makes her a high probability carrier for X-linked muscular dystrophy. P. S., a maternal cousin of G. S., expressed interest in determination of carrier status for the purpose of family planning. Results in 2 independent hybridization studies with probe 8 are consistent with low probability for P. S. to be carrier. The dosage ratios (Table 4) were 0.71 (3.7,3.8/3.1) and 0.70 (10/7) compared to the same band ratios in the control of 0.79 and 0.64.

Family 4. In family 4 the DMD male, J. B., was found to be deleted by multiplex PCR in the area of primer h. cDNA analysis with probe 8 confirmed this deletion by failure to hybridize to the 3.1 kb fragment (Figure 14, lane 5). The hybridization patterns of the mother (M. B.), 2 sisters (J. B. and P. B.) and a control are shown in Figure 14 in lanes 6, 7, 8 and 10 respectively. The mother and sisters were evaluated by densitometry for evidence of reduced dosage in the 3.1 kb band. Dosage ratios for the 3.7,3.8/3.1 kb fragments are shown in Figure 15. The increased dosage ratio in the mother (M. B.) of 1.91 (Figure 15, B) and in one sister (P. B.) of 1.86 (not shown) were evidence for haploid dosage of the 3.1 kb fragment with approximately 50 percent reduction in band intensity

Figure 14. Autoradiogram 90-4, Hybridization with DMD cDNA Probe 8



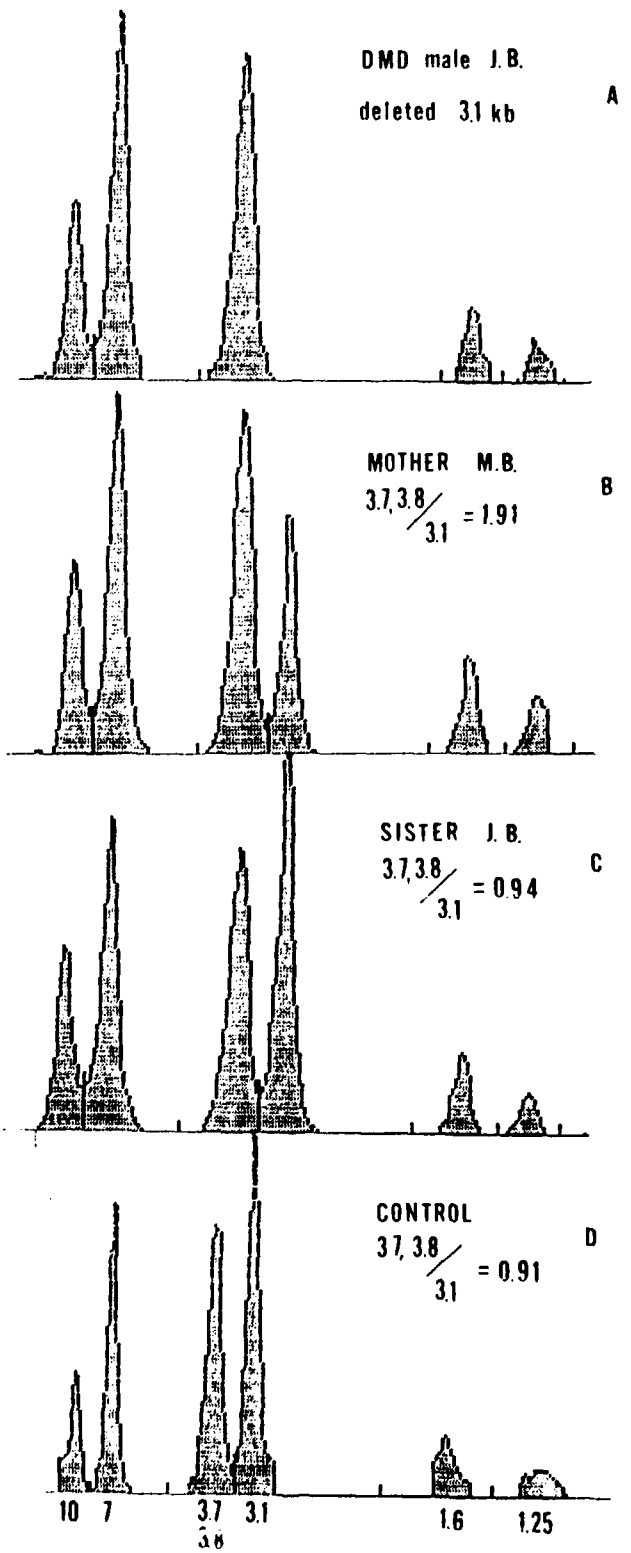


Figure 15. Family 4, densitometric scan

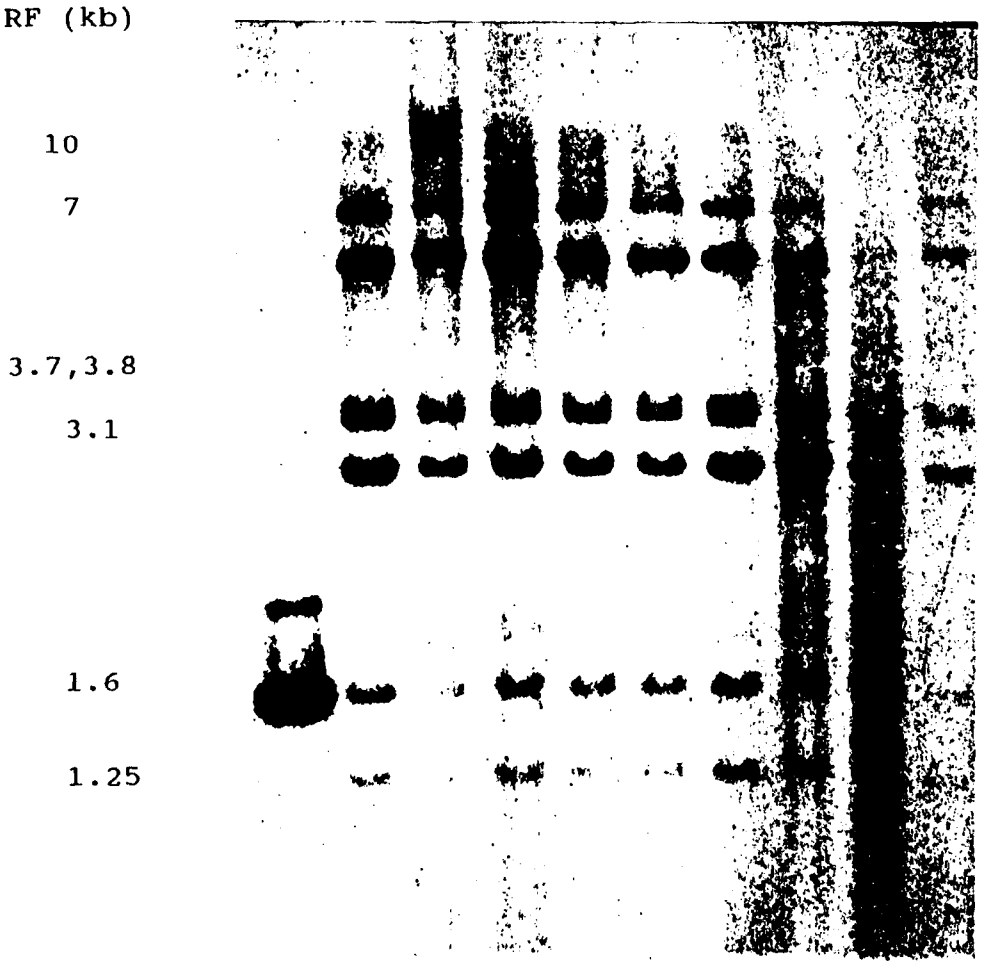
relative to the 3.7,3.8 kb fragments when compared to the control ratio of 0.92 (Figure 15, D). The sister J. B (Figure 15, C) demonstrates normal diploid dosage of the 3.1 kb fragment having a 3.7,3.8/3.1 ratio of 0.92. The assessment of high probability carrier status for the mother (M. B.) and sister (P. B.) and low probability carrier status for the sister (J. B.) by cDNA studies is in agreement with previous results which showed repeated elevations in serum CK for the mother (225 U/L) and P. B. (478 U/L) with reduced lymphocyte capping observed in both females (132). J. B. had normal serum CK (47 U/L) but also showed reduced capping values. In RFLP linkage studies J. B. was shown to receive the opposite fragment from her brother (J. B.) and sister (P. B.) which made it unlikely that she was a carrier (125, 126). Because cDNA hybridization analysis provides direct assessment of the gene, it eliminates the ambiguity inherent in the other methods of carrier analysis described.

Family 5. Similar correlation between cDNA hybridization studies and previous results for serum CK and lymphocyte capping was observed in family 5. A deletion in the area of primer f was detected by the 6 primer multiplex PCR in the DMD male, J. F. Analysis with cDNA probe 8 confirmed and extended J.F.'s deletion to include the 10, 3.7,3.8, 3.1, 1.6 and 1.25 Kb fragments. Hybridization

studies with probe 8 were performed in the mother (E. F.) and 3 sisters (L. F., B. F. and S. F.) shown in Figure 16 lanes 3, 4, 5 and 6 respectively. Normal nondeleted controls are present in lanes 2 and 7 on this autoradiogram. Dosage ratios were calculated between the 7 kb fragment which was not deleted in J. F. and the 3.1 or the 3.7,3.8 kb fragments in the females. In the densitometric scan shown in Figure 17 (B) the mother (E. F.) demonstrates elevated values for both ratios (7/3.1 = 2.69 and 7/3.7,3.8 = 2.23) when compared to the normal control (1.80 and 1.38) and the sister S. F. (1.80 and 1.58). The other sisters, L. F. and B. F., also had elevated values for both dosage ratios (data in Table 4). The increased values for the ratios in the mother and 2 sisters L. F. and B. F., due to the reduced band intensities of the 3.1 and the 3.7,3.8 kb fragments, indicates haploid dosage. The data indicate that the mother and sisters L. F. and B. F. have high probabilities of being carriers while S. F. has only a low probability.

Previous studies had shown elevations in serum CK for E. F. (333 U/L), L. F. (1006 U/L) and B. F. (771 U/L) while S. F.'s CK fell within the normal range (34 U/L). Lymphocyte capping values were reduced in all but S. F., who showed normal values. Unfortunately RFLP linkage analysis showed that all 3 sisters had received the same fragment as their brother, which created some uncertainty in the interpretation of carrier status in S. F. (125, 126).

Figure 16. Autoradiogram 90-2, Hybridization with DMD cDNA Probe 8



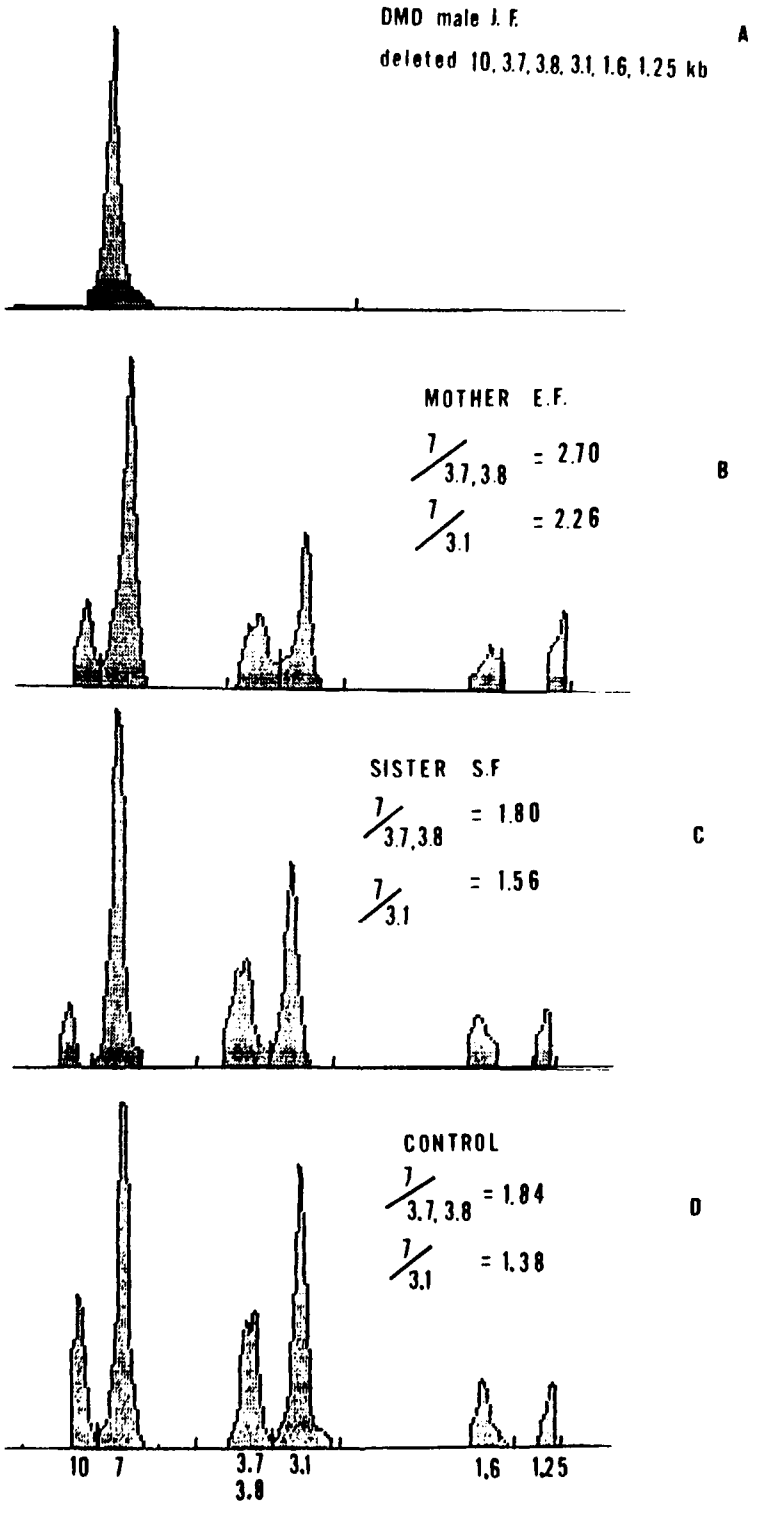


Figure 17. Family 5, densitometric scan

An explanation for this apparent discrepancy was either possible recombination in S. F. such that she was not a carrier or Lyonization, which could account for the normal CK and capping values but would result in positive carrier status. The advantages of cDNA hybridization analysis with densitometric quantitation are evident in this case by allowing more accurate determination of carrier status to be made.

There may be cases in which the only deletion present is in one of the 2 comigrating fragments, 3.7 or 3.8. Such is the case of DMD male D. D., who has a very small deletion of only the 3.7 kb fragment by hybridization with cDNA probe 8 (Figure 14, lane 2). D. D's deletion was not detected in the multiplex PCR screen since primers are not currently available for exon 50. Evidence for a deletion of the 3.7 kb fragment was provided by a comparison of band ratios obtained by densitometric analysis of hybridization signal for 3.7,3.8/3.1 kb fragments in D. D. and a male known not to be deleted for the 3.7 or 3.8 bands. A reduced ratio was found in D. D. (0.35) while the control demonstrated a value of 1.0 which is on the upper end of the normal reference range for this ratio (0.59-1.03). Another male, M. M., who is deleted for only the 3.8 kb fragment also showed a reduced dosage ratio of 0.30. Interpretation of dosage in males is more straightforward since they are hemizygous. As previously mentioned the ratio of 3.7,3.8/3.1 is difficult to interpret in females when only

one of the comigrating fragments is deleted. It would be important to establish a range of values for this dosage ratio in obligate carrier females known to be deleted for just one of the fragments (3.7 or 3.8 kb). To date values for this type of deletion have been limited to one obligate carrier female (M. M.). Two attempts have been made to evaluate D. D's mother (C. D.), an obligate carrier with 3 affected sons, who represents another case in which only one of the comigrating fragments is deleted. Unfortunately there was poor hybridization with probe 8 possibly due to deterioration of her DNA (Figure 14, lane 3). The maternal aunt of D. D., R. H., has been evaluated by hybridization with probe 8 (Figure 14, lane 4) and shows a 3.7,3.8/3.1 ratio of 1.05. The ratio suggests low probability of being a carrier; however, only a limited interpretation is possible since there are currently no values for the mother, C. D. Future analysis of a fresh DNA sample from the mother (C. D.) and the maternal grandmother should assist in providing a more certain interpretation of carrier status for R. H.

Reference Intervals for Dosage Ratios

Reference intervals for dosage ratios obtained in probe 8 hybridization studies were calculated for controls and females determined to be low probability carriers. There is presently an insufficient number of high probability carriers to establish ranges for this group.

In Figure 18 the distribution of values for control and low probability carriers for the 3.7,3.8/3.1 kb ratio is compared to the values for high probability carriers, deleted for fractions 3.7,3.8 kb. The reference interval for 10 controls for this ratio was determined to be 0.58 to 1.03 with a mean of 0.81 and standard deviation (SD) of 0.11 which is comparable to the range for 19 low probability carriers of 0.69 to 1.05, with a mean of 0.87 and an SD of 0.08. The high probability carriers who demonstrated haploid dosage for the 3.7,3.8 kb fragments had approximately 50 percent reduced dosage ratios compared to controls. The 3.7,3.8 /3.1 kb ratio has provided the most useful values for making carrier determinations since these bands consistently transfer well, producing good intensities for densitometry, and are present in approximately equal amounts in the majority of nondeleted individuals.

The reference interval for the 10/7 dosage ratio is shown in Figure 19 comparing the distribution of control and low probability carrier females to those of females assessed to be high probability carriers. The range of 0.34 to 0.62 (mean, 0.47, SD, 0.07) for the control group (n = 7) is comparable to that of the low probability carrier range of 0.31 to 0.71 (mean, 0.51, SD, 0.1, n = 14). The ratios for the high probability carriers (0.18, 0.22 and 0.26) are half the control ratios which is consistent with haploid dosage for the 10 kb fragment in these individuals.

Figure 18. Dosage Ratio of 3.7, 3.8 kb to 3.1 kb Band Intensities

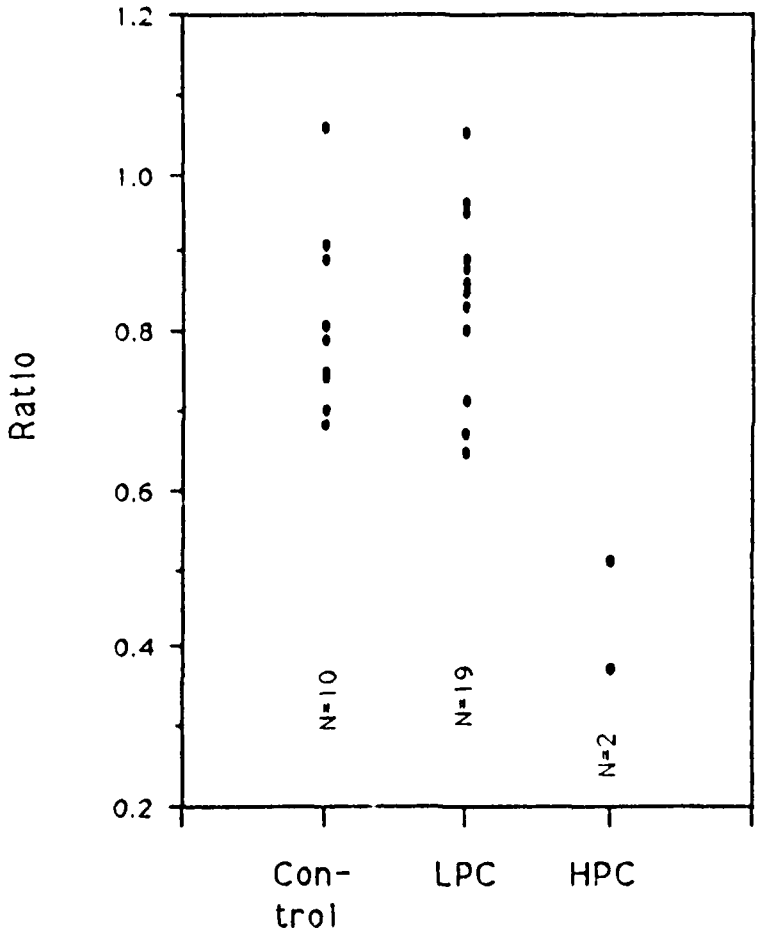
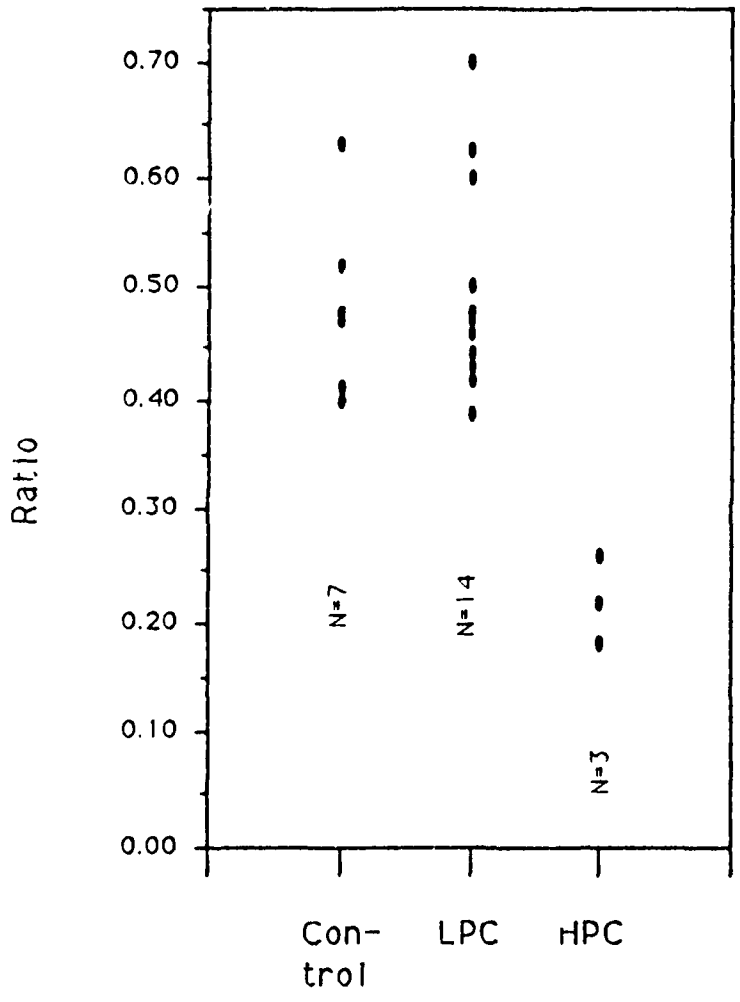


Figure 19. Dosage Ratio of 10 kb to 7 kb Band Intensities



The utility of the 10/7 ratio is sometimes limited by the problem of reduced transfer of the 10 kb fragment which is evident on visual inspection of band intensities on autoradiograms. In initial hybridization studies a depurination step was not performed since fragments greater than 15 kb were not expected with probe 8 and smaller fragments should transfer well without depurination. Because of the problems encountered with the 10 kb fragment and the appearance of possible junction fragments greater than 15 kb, a depurination step was added in later experiments. Adding the depurination step improved the 10 kb transfer in one case (autoradiogram, Figure 20) but did not result in improved transfer in another case (autoradiogram, Figure 16). The recurrent difficulty with the 10/7 ratio demonstrates the need for using an additional band ratio where possible to increase the reliability in carrier determinations.

Precision Studies

The occurrence of such variation as seen in the 7/3.1 and 7/3.7,3.8 band ratios among different individuals is also observed in the ratios for a specific control DNA present on 6 independent autoradiograms. Table 5 reviews the results of precision measurements for this control sample in which high coefficients of variation (CV) for all fractions indicate significant differences in band intensities from blot to blot. Because of the extreme

Figure 20. Autoradiogram 90-6, Hybridization with DMD cDNA Probe 8

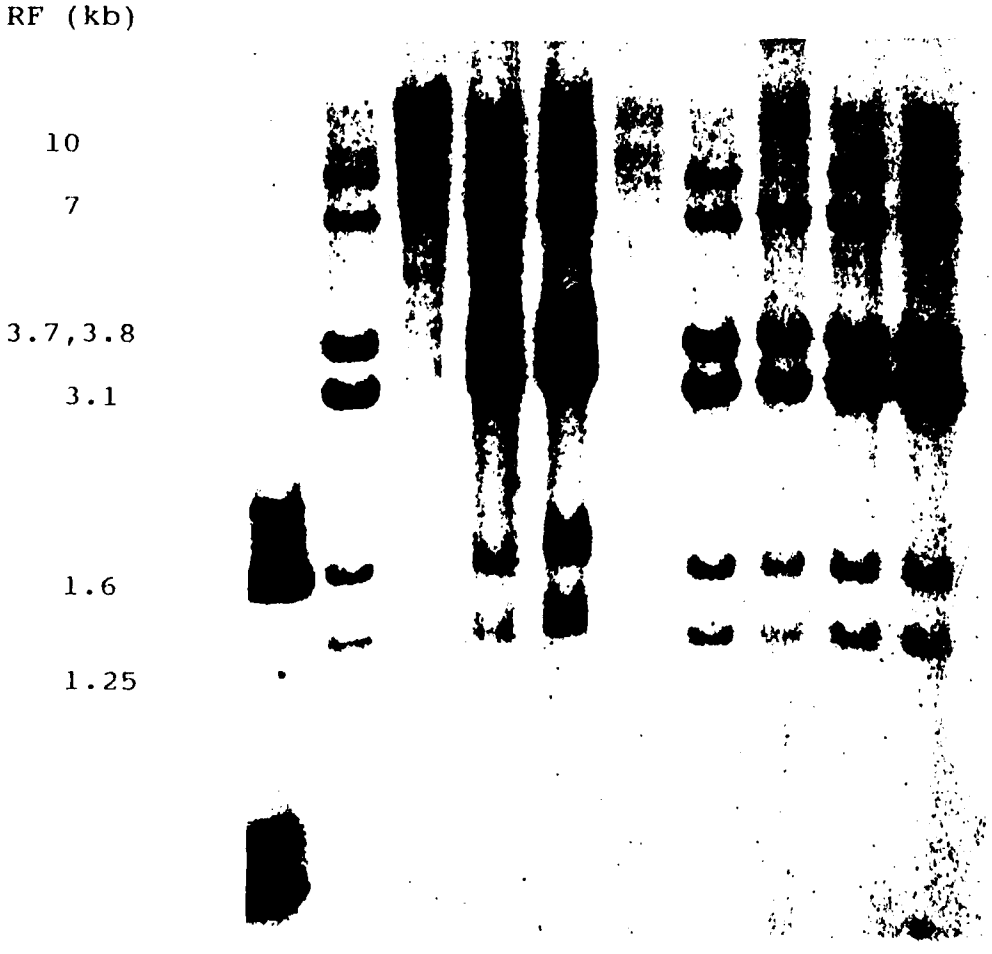


Table 5

Interblot Variation of Control DNA Restriction Fragment Intensities

Restriction fragment	10kb	7kb	3.7, 3.8kb	3.1kb	1.6kb	1.25kb
Standard deviation	1.5	2.2	2.8	4.6	2.3	1.4
Mean	9.2	20.7	28.2	32.0	7.1	2.9
CV	16.7	10.8	10.1	14.4	33.0	50.3

Values for control DNA were obtained from 6 independent autoradiograms. Values are calculated as relative percent of total.

8.3	17.9	24.6	36.8	8.4	4.1
6.7	19.2	29.3	35.9	5.9	3.0
9.4	22.0	28.7	30.2	8.5	1.3
9.4	23.4	32.6	28.5	3.8	2.3
10.2	19.2	28.3	35.0	5.8	1.6
11.2	22.6	25.6	25.4	10.3	4.9

variation in intensities of the 1.6 and 1.25 kb bands they have not been used to calculate dosage ratios.

Since dosage ratios are used in interpretation of carrier status rather than band intensities, the interblot variability of dosage ratios was determined. Data are presented in Table 6 which indicate significant imprecision of ratios for the control DNA present on different blots.

Table 6

Interblot Variation of Control DNA Dosage Ratios

Ratio	10/7 kb	3.7,3.8/3.1 kb
SD	0.06	0.15
Mean	0.45	0.90
CV	13.3%	16.7%

Values represent the ratio of restriction fragment band intensities for control DNA on 6 independent autoradiograms.

0.46	0.67
0.35	0.82
0.43	0.95
0.40	1.14
0.53	0.81
0.50	1.00

A CV of 13.3% was observed for the 10/7 kb ratio while a CV of 16.7% was seen for the 3.7,3.8/3.1 kb ratio. The large CV for the dosage ratios between blots emphasizes the need for using a control ratio from the same blot for dosage comparisons in carrier determinations. Optimally several controls should be included on a blot to provide more than one set of ratios for comparison to those of suspected carriers.

In Tables 7 and 8 results of 2 independent precision studies are presented. The values were obtained from 10 (Table 7) or 12 (Table 8) replicate measurements of band intensities in 2 different DNA samples. The autoradiogram was removed from the densitometer and repositioned between each replicate scan, and the Electrophoresis Data Center was reprogrammed between each scan to reset scan parameters. There is good precision (less than 5%) for all fractions with 3 exceptions. The 1.6 and 1.25 kb fragments have greater variability due to the weaker band intensities which makes them more difficult to quantitate accurately. The 10 kb fragment in precision study 2 (Table 8) shows a coefficient of variation of 8% which may also be attributed to the reduced intensity of this fraction in this sample.

The variability of dosage ratios was calculated for both precision studies 1 and 2. As indicated in Tables 9 and 10, the densitometer has good precision on replicate measurements as seen in the CVs for 10/7 kb (4.3% [study 1] and 6.3% [study 2]) and for 3.7,3.8/3.1 kb (2.4% for both study 1 and 2).

While there is good precision in repeat measurements of a DNA sample from the same densitometric scan, there can be significant variation in dosage ratios for a given sample due to differences in baseline settings. To investigate this source of variation densitometric scans were performed on a DNA sample using different baseline settings for each scan. The 10/7 dosage ratios obtained in 3 measurements

Table 7

Precision Study 1: Restriction Fragment Intensities

Restriction fragment	10kb	7kb	3.7,3.8kb	3.1kb	1.6kb	1.25kb
Standard deviation	0.3	0.2	0.2	0.5	0.3	0.3
Mean	7.7	11.0	31.0	36.4	8.7	5.1
CV	3.5	1.9	0.8	1.4	3.8	6.0
	Values are for 10 replicates of each restriction fragment, same blot. Values are calculated as relative percent of total.					
	7.9	11.0	31.0	36.1	8.7	5.3
	7.7	11.1	31.1	35.9	8.8	5.3
	7.4	11.4	31.4	35.7	9.0	5.2
	7.2	10.7	31.1	36.5	9.0	5.5
	7.6	10.8	31.3	36.3	8.7	5.2
	7.9	10.7	30.8	36.5	8.8	5.3
	7.9	11.2	30.5	36.8	8.6	5.0
	8.0	11.0	31.2	37.6	7.8	4.4
	7.6	11.0	31.0	36.3	8.8	5.2
	8.0	10.8	31.0	36.5	8.6	5.0

Table 8

Precision Study 2: Restriction Fragment Intensities

Restriction fragment	10kb	7kb	3.7,3.8kb	3.1kb	1.6kb	1.25kb
Standard deviation	0.4	0.9	0.5	0.7	0.6	0.2
Mean	5.5	17.2	29.1	34.4	10.4	3.5
CV	8.0	5.1	1.6	2.0	5.7	5.7
	Values are for 12 replicates of each restriction fragment, same blot. Values are calculated as relative percent of total.					
	5.7	17.9	28.5	33.4	10.8	3.7
	5.2	15.9	29.3	35.0	10.9	3.7
	6.0	18.6	29.1	33.7	9.4	3.2
	5.5	16.9	28.1	34.6	11.1	3.8
	5.1	16.4	29.7	35.4	10.1	3.4
	4.6	16.3	29.9	35.3	10.5	3.3
	5.2	18.0	29.4	33.8	10.0	3.5
	5.2	16.2	29.0	34.9	11.2	3.7
	6.2	17.5	28.8	34.3	9.9	3.2
	5.7	17.0	28.7	34.4	10.7	3.4
	5.7	18.0	29.2	33.7	9.9	3.5
	5.7	17.8	29.2	34.0	9.6	3.7

Table 9

Precision Study 1: Variability of Dosage Ratios

Ratio	10/7 kb	3.7,3.8/3.1 kb
SD	0.03	0.02
Mean	0.70	0.85
CV	4.3%	2.4%

Values represent the ratio of restriction fragment band intensities obtained from each replicate measurement in precision study 1.

0.72	0.86
0.69	0.87
0.65	0.88
0.67	0.85
0.70	0.86
0.74	0.84
0.71	0.83
0.73	0.83
0.69	0.85
0.74	0.85

Table 10

Precision Study 2: Variability of Dosage Ratios

Ratio	10/7 kb	3.7,3.8/3.1 kb
SD	0.02	0.02
Mean	0.32	0.85
CV	6.3%	2.4%

Values represent the ratio of restriction fragment band intensities obtained from each replicate measurement in precision study 2.

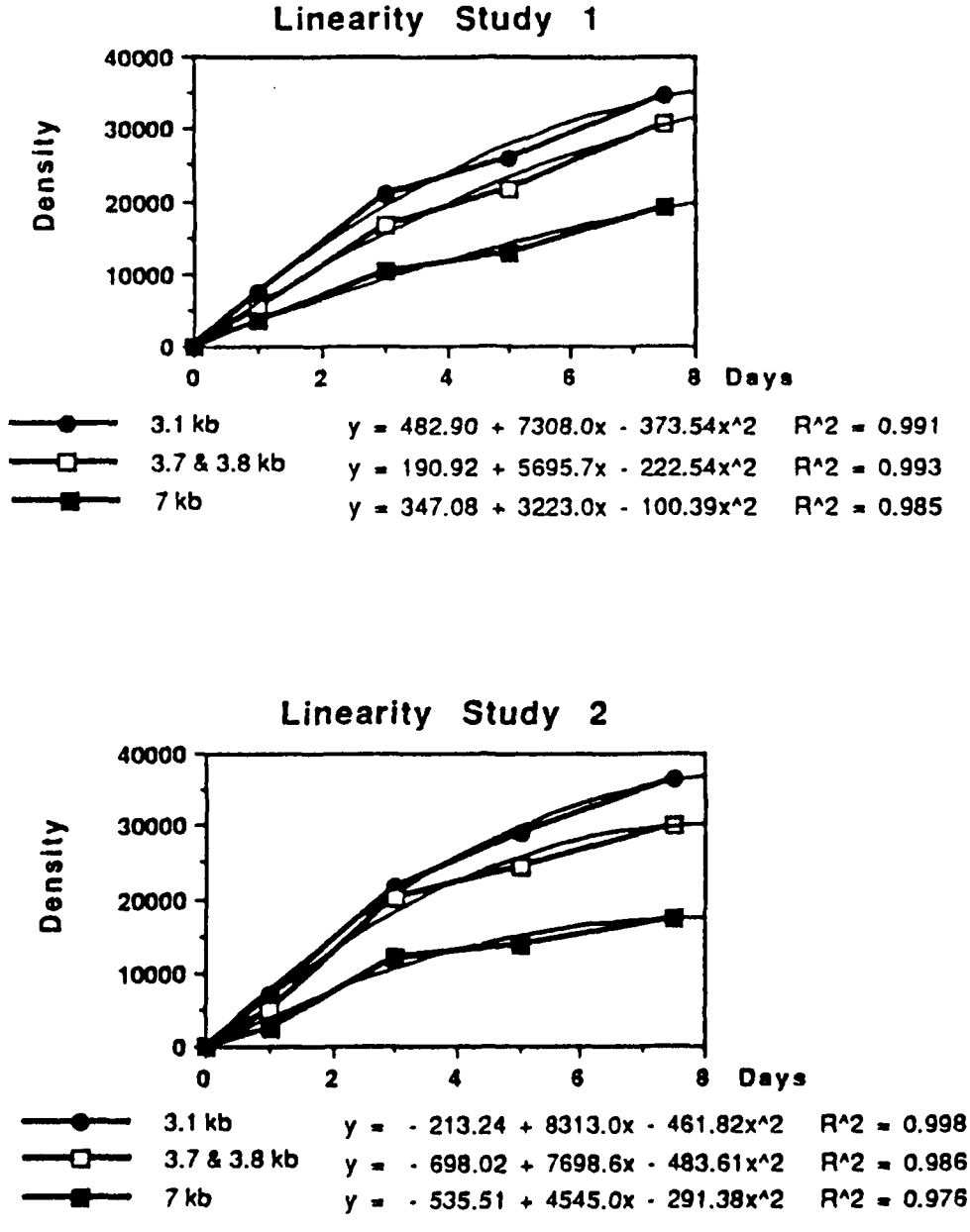
0.32	0.85
0.33	0.84
0.32	0.86
0.33	0.81
0.31	0.84
0.28	0.85
0.29	0.87
0.32	0.83
0.35	0.84
0.34	0.83
0.32	0.87
0.32	0.86

were 0.60, 0.42 and 0.60. In this instance the interpretation of diploid dosage for the 10 kb fragment would be made for all three values; however, there may be instances in which differences in baseline settings could produce a different interpretation for carrier status. It is essential to be consistent in establishing baselines during the editing procedure particularly in family studies on the same blot.

Linearity Studies

There are a number of reports in the literature in which gene dosage is determined from restriction fragment intensities. The general approach is to estimate the dosage of the bands by visual inspection. Several investigators (96, 101, 102) have reported densitometric evaluation of band intensities; however, there is no available data to establish the validity of the method for carrier determinations. Since there was a need for validation of dosage analysis by densitometry, studies were carried out to investigate various components associated with performance reliability. Results of 2 independent studies to evaluate the linearity of the densitometric band intensities with increased exposure of the autoradiogram are shown in Figure 21. Data are presented for the 3.1, 3.7,3.8 and 7.0 kb restriction fragments detected by cDNA probe 8. As described in Materials and Methods the band intensities were calculated as integrals relative to a preset internal

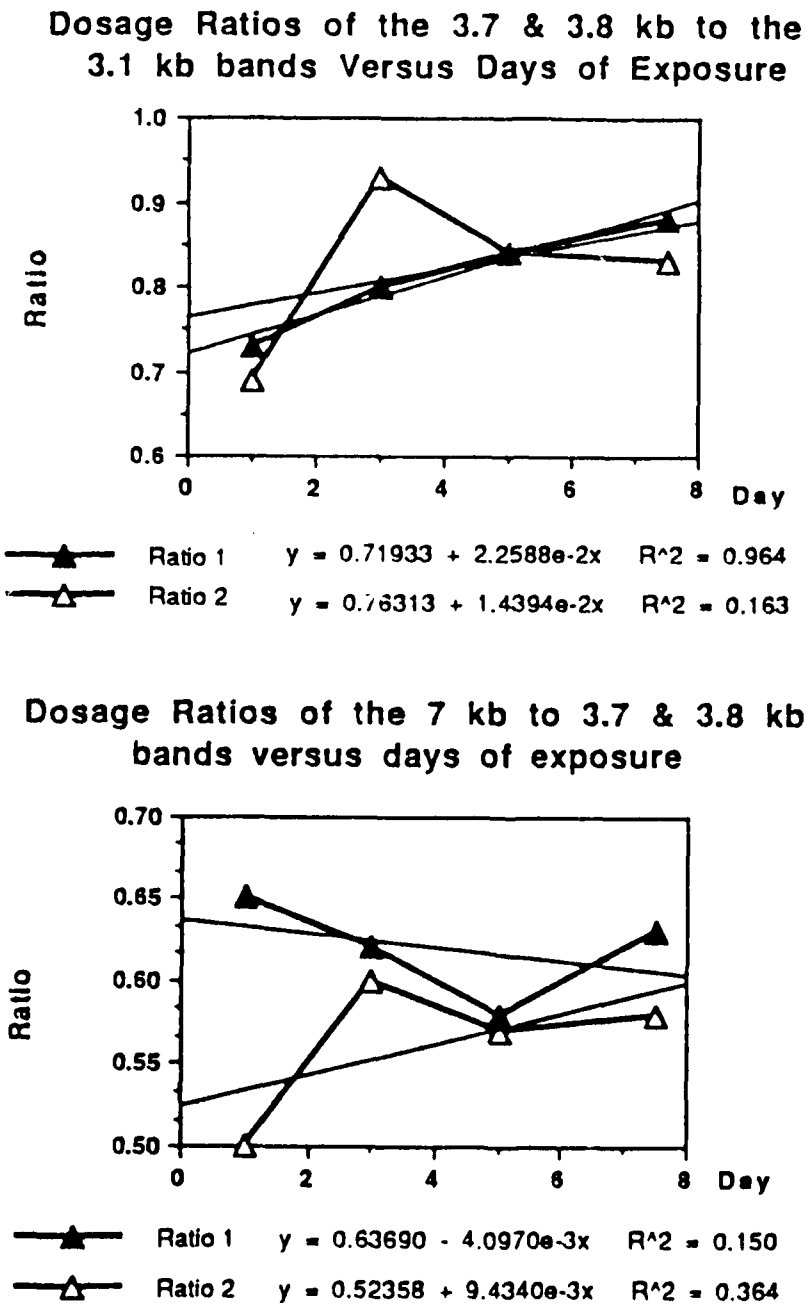
Figure 21. Linearity of Restriction Fragment Band Intensity versus Exposure Time



standard. The integral values obtained for each restriction fragment for exposure times of 1, 3, 5 and 7.5 days were corrected for disintegration. The factors used for correction were derived from values for decay of ^{32} P (128) using the time at which 50% disintegration occurred for each of these exposure times: for 1 day, 50% occurred at 12 hours, factor = 1.025; for 3 days, 50% occurred at 1.4 days, factor = 1.070. For the 5 and 7.5 day exposure times, 3 days were added to the 50% disintegration time in calculating a correction factor since the exposure for 5 and 7.5 days was started 3 days after the first set of films were removed: for 5 days, 50% occurred at 2.75 days plus 3 days = 5.2 days, factor = 1.287; for 7.5 days, 50 % occurred at 3.25 days plus 3 days = 6.25 days, factor = 1.354. By correcting with a factor for the disintegration midpoint, an average value is obtained, while use of the factor at the disintegration endpoint would result in an overestimation.

While polynomial equations of several degrees were applied, a polynomial of the second degree provided the best fit for the data in all cases. The relationship between the integral values of band intensities and autoradiogram exposure is not absolutely linear; therefore, the relationship of the dosage ratios and exposure time was evaluated. Dosage ratios for the 3.7,3.8/3.1 kb bands and the 7/3.7,3.8 kb bands at 4 exposure times are presented in Figure 22. While small variations between dosage ratios are observed at different exposure times, they are not

Figure 22. Dosage Ratios versus Exposure Time



sufficient to alter the interpretation with respect to carrier status. For both data sets (ratio 1 and ratio 2) the 3.7,3.8/3.1 kb ratios fall within the control reference range (0.59-1.03) and would predict low probability of being a carrier. Although there is some correlation between the dosage ratios and exposure time for the ratio 1 data set, the slight increase in values with increased exposure does not change the interpretation. Similar findings were obtained with the 7/3.7,3.8 kb ratios which exhibit slight variation with different exposure times, yet still produce the same interpretation of low probability carrier status.

Linearity was demonstrated for the densitometer over the range of values found for autoradiogram band intensities, which should exclude the possibility that the observed deviations from linearity were caused by band intensities exceeding the linear range of the instrument. The lack of linearity might also be explained by characteristics of the X-ray film with radiation exposure.

The results obtained in the linearity studies provide increased assurance in the reliability of densitometric quantitation of gene dosage and validate the method as a means of diagnosing carriers of X-linked muscular dystrophy.

Conclusions

To fulfill the final objective of this project, criteria have been established for performance and interpretation of densitometric scans of restriction fragment band intensities on autoradiograms for DMD carrier determinations.

One of the essential requirements for a successful scan is a good quality blot. The DNA sample must be adequately digested and transfer to the nylon filter should be complete, providing well resolved bands without smearing. The comparison of band ratios rather than single band intensities circumvents the differences in DNA transfer and labeling from lane to lane.

In order to minimize problems due to shifts in the baseline, contiguous bands should be used when possible for calculation of dosage ratios. Using bands of approximately equal intensity facilitates the interpretation of ratios since a 50% decrease in dosage is more readily apparent. Increased reliability of the carrier determination may be obtained by using more than one dosage ratio when possible.

While reference ranges established for a specific band ratios should be useful in the interpretation, it is necessary to compare the test ratio with a normal, nondeleted control ratio from the same autoradiogram since differences in transfer occur between blots. Reference ranges determined in this study were calculated using two standard deviations from the mean; therefore, a patient ratio falling within the reference range should be in the 95% confidence interval for a given reference range. As indicated in the family studies, additional information such as RFLP linkage data and CK and lymphocyte capping values should be considered in making a final assessment of the probability of carrier status. In order to validate quantitation by densitometric analysis as a means of determining carrier status, it is imperative to confirm the method using obligate carrier females who demonstrate reduced dosage compared to nondeleted controls.

To increase confidence in the results for carrier status precision studies using replicate scans of a lane should be performed. To further validate the densitometric method performance of linearity studies between band intensity and time of autoradiogram exposure is recommended. The dosage ratios obtained at each exposure should fall within the same reference range and provide the same interpretation for carrier status.

With the major developments in muscular dystrophy research, it is now possible to make direct assessment of

the gene for dystrophin. While there is currently no cure for this devastating disease, there is potential for reducing the incidence by improved methods of carrier identification. A diagnostic approach is described which begins with deletion screening of affected males using multiplex PCR and extends to carrier assessment in female relatives by densitometric analysis of restriction fragments detected by cDNA probe hybridization.

There are reports of dosage analysis being used for carrier determinations. No information regarding the validity of using densitometry for gene dosage was available. The results described in the present study for precision, linearity and correlation with previous linkage data provide support for the reliability of the densitometric method of dosage analysis. It is anticipated that improved methods of dosage analysis utilizing the PCR for gene haplotyping will provide a more straightforward approach and facilitate the diagnosis of DMD carriers.

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